THERAPY OF VIRUS-INFECTED PLANTS BY HEAT TREATMENT.

by

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To the best of my knowledge, this thesis contains no material which has been accepted for the award of a degree or diploma by any University, nor any copy of material previously published or written by any other person without specific reference being made in the text.

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

SUMMARY

Tomato aspermy virus (TAV) was found infecting more than 90% of chrysanthemum plants collected from several gardens in the Hobart district. The virus is readily transferred by sap inoculation to tobacco (Nicotiana tabacum var. Hickory Prior) in which it causes a severe systemic disease, and real relative concentrations of infective particles in tobacco plants can be measured reliably on Chenopodium amaranticolor, a local lesion host, if certain procedures are adhered to.

Tobacco plants may be freed from infection by growing them at a constant temperature of 36°C for between 5 and 30 days. The percentage of cures effected is generally greater if cuttings are taken from plants immediately after treatment and rooted in a mist propagation unit.

A decreased rate of viral synthesis is one reason for the effectiveness of the method. Under normal conditions of heat treatment, virus multiplication could never be detected although some indirect evidence, from results with cytokinin applications to treated plants, suggested that limited multiplication still occurred. However, measurable synthesis did take place if heated plants were treated with actinomycin D. These observations, together with some additional information on rates of TAV accumulation in plant tissue under various conditions, support a concept of competition between the virus and its host for substrates and access to ribosomes. As ribosomal concentration falls markedly in plants undergoing heat treatment concomitant with an increase in host protein synthesis, an hypothesis is

advanced that viral RNA competes poorly with host messenger RNA for the limited numbers of available ribosomes on which to synthesise their respective proteins. This concept may also be used to account for the successes in obtaining virus-free material from infected plants by meristem culture.

The rate of virus inactivation in whole plants at 36^{o}C is much slower than the rate in expressed sap, and faster than the rate in purified virus preparations. Also, the kinetics of inactivation both 'in vivo' and in expressed sap do not fit a reaction of the first order, but one of a higher order, showing that inactivation at high temperature is not solely a direct thermal effect. Large increases in the levels of two enzymes, polyphenoloxidase and ribonuclease, occur during heat treatment and these may directly inactivate TAV. The extent of increase of these heat-induced enzymes may vary greatly with the species and variety of tobacco, thus providing an explanation for many past observations and suggestions implicating a role of the host in heat treatment therapy.

Several observations indicated that the cytokinin concentration falls in heated plants. This results in an almost complete cessation of mitosis as well as a decrease in the ionic strength of the cell sap concomitant with a large increase in cell volume. The pH of the cell sap also falls markedly during heat treatment. These changes probably reduce the stability of TAV. The observations on alterations in cell size and mitotic activity are taken to negate the commonly advanced suggestion that heat treatment

may be successful because the plant "grows away" from the virus.

The results, and some conclusions drawn from them, offer several different mechanisms which may be important in achieving virus therapy by heat treatment. They allow one to predict the types of virus infections which might be cured by heat treatment, and suggest that exogenous application of certain chemicals to plants may be useful in increasing both plant survival and the chances of virus cure in "difficult plants." A more rational approach to virus therapy by heat treatment should therefore be possible.

LITERATURE REVIEW.

I INTRODUCTION

Many clones of vegetatively propagated plants are universally infected with one or more viruses, and healthy material can therefore be obtained only by eliminating them from the existing infected stocks (Hollings, 1965a). Three methods have been used to achieve this end. They are the use of chemicals, the use of high temperature, and the culture of tissue not systemically invaded by virus. The methods were pioneered by Stoddard (1942), Wilbrink (1923) and Morel & Martin (1952), respectively. Combinations of the three methods have also been used successfully in recent years (e.g. Campbell, 1962).

This review concerns the use of high temperature to obtain virus-free plants from infected sources, with particular emphasis on the mechanisms involved. The subject has been reviewed several times in recent years (Baker, 1962; Hollings, 1965a; Hollings & Stone, 1968; Houten et al., 1968; Kassanis, 1957a; Kassanis, 1967; Kassanis & Posnette, 1961; Nyland, 1964; Nyland & Goheen, 1969; Posnette, 1961; Stubbs, 1963a, 1966). However, these reviews were chiefly concerned with the practical aspects of heat treatment.*

The use of heat treatment to obtain virus-free stock developed slowly following Wilbrink's original report

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* heat treatment is used in preference to "thermotherapy" throughout this review, as the latter connotes that the high temperature 'per se' is responsible for virus inactivation.

(Bawden, 1964a), until work at the East Malling Research Station during the 1950's demonstrated that several widespread and economically important virus diseases of berry, pome and stone fruits were amenable to heat treatment (Ellenberger, 1960; Posnette, 1953; Posnette & Cropley, 1956, 1958; Posnette et al., 1953, 1962).

These reports stimulated world-wide interest in the subject, and by 1965 Hollings was able to list 91 virus diseases which had been successfully heat-treated. Australian workers have been among those actively engaged in this field; most of their work is unpublished and details of it have been collected therefore, and set out in Appendix 1.

The production and distribution of virus-free Cambridge Favourite strawberry plants in England has led to an increased production estimated to be worth £1 million annually (Anon, 1968). This is but one example of the economic significance of heat treatment, and rightful emphasis on practical aspects of the subject has meant that little time has been available to investigate the mechanisms by which successful therapy is achieved.

II METHODS OF HEAT TREATMENT

Treatments in both hot water and hot air environments have been used to obtain virus-free plant material. Where compared, the former method was found less satisfactory (Chambers, 1954; Kegler, 1959; Posnette, 1953; Posnette et al., 1962).

(a) hot water

The use of hot water has been limited almost

entirely to dormant material as growing plants are rapidly killed in hot water. For example, Posnette (1953) found that strawberry plants survived only 7 hours in water at 35°C whereas they survived up to 8 days in air at 37°C. That this is probably due to the much higher specific heat and specific conductivity of water compared to air is suggested by the results of Kegler (1959) who found that the internal temperature of apple shoots reached that of the water in which they were immersed within one minute, whereas a temperature difference of 25C° existed between the centre and surface of shoots 10 minutes after placement in a warm air environment.

Treatments are normally carried out in the region of 50-55°C for from several minutes to several hours. Extremely good temperature control is achieved if the volume of water is large in proportion to the material being treated. One outstanding success with hot water has been the routine treatment of sugar cane setts at 50°C for 2 hours to control ratoon stunt in Queensland (Steindl & Hughes, 1953).

(b) <u>hot air</u>

Generally, treatments with hot air have been applied to growing plants within the range of 34-40°C for periods of from several days to several weeks. The growth cabinets used by different workers for treatment of plants have varied from very simple constructions which control temperature within about 2C° of that desired (e.g. Posnette & Cropley, 1958) to very sophisticated cabinets which control temperature within 0.1C°

and allow for continual air exchange (e.g. Stubbs, 1963b). Some workers have stressed the necessity for a steady high temperature during treatment (e.g. Kunkel, 1936), whereas others have achieved success when they fluctuated temperature during treatment in an attempt to aid plant survival (e.g. Brierley, 1964; Mellor & Fitzpatrick, 1961). The fact that several viruses have been inactivated in plants growing naturally in high temperature environments indicates that a steady high temperature is not essential for the elimination of all viruses from their infected hosts (Frazier et al., 1965; Kunkel, 1936; Thirumalachar, 1954).

Linacre (1967) has compiled extensive evidence to indicate that plants control their transpiration rate and metabolic processes so that their internal leaf temperature approaches an "equality temperature" which is in the vicinity of 30°C for most plants. No leaf temperature measurements have been reported for plants undergoing heat treatment, nor have the critical temperature-time values necessary for successful virus inactivation. The relative humidity, rate of air circulation and inflow of solar radiation are all important in controlling leaf temperature. Differences between these factors may explain why some workers have reported marked growth of a plant species, such as apple, during treatment at a particular air temperature (e.g. Campbell, 1962) while others have not (e.g. Johnstone, unpublished data).

III MECHANISM OF INACTIVATION BY HOT WATER TREATMENT

Most viruses successfully inactivated by hot water treatment are not sap-transmissable and therefore, their thermal inactivation points are unknown. However, the temperature-time combinations used (50-55^{o}C for from several minutes to several hours) are within the region where most plant viruses are destroyed 'in vitro', and Hollings (1965a) suggested that hot water therapy probably results from a direct thermal inactivation of virus. Kassanis (1957a) reached the same conclusion on the basis of the rapidity with which inactivation is achieved. Their conclusions on the concept of a direct thermal effect are supported also by the evidence that some seed borne viruses are destroyed only by heating seed to temperatures in excess of that necessary to destroy the viruses 'in vitro' (Broadbent, 1965; Megahed & Moore, 1969; Rader et al., 1947).

IV MECHANISM OF INACTIVATION BY HOT AIR TREATMENT

Gregory & Sen (1937) first showed that the proteins in barley leaves are continually being synthesised and degraded, and that the concentration of protein in the leaves at any time reflects the balance of these two activities. This phenomenon appears to be universal, concurrent protein synthesis and degradation having been demonstrated in many different plants (e.g. Hellebust & Bidwell, 1964; Racusen & Foote, 1960).

Because of the similarity in mechanism between viral and plant protein synthesis (Bosch et al., 1966; Weissmann et al., 1966), it would appear probable that plant viruses

are also normally in a constant state of synthesis and degradation. Evidence is readily available to show that plant viruses multiply within their hosts and that, under certain experimental conditions, they are degraded 'in vivo'. However, technical problems make it difficult to show that both processes occur simultaneously under any one set of environmental conditions. To my knowledge, the only direct evidence of this phenomenon occurring with plant viruses comes from the work of Harrison (1956) who showed that tobacco necrosis virus may both multiply and be degraded in bean leaf tissue at 30°C, and that of Kassanis (1957b) who found that a common strain of tobacco mosaic virus could both multiply and be degraded in systemically infected tobacco leaves at 36°C. Notwithstanding this meagre evidence, it is generally considered that viral synthesis and degradation proceed concurrently within the infected plant under most experimental conditions (Bawden, 1964a; Harrison, 1956; Kassanis, 1957a).

Kassanis & Posnette (1961) postulated that the two processes are affected differently by temperature and that heat treatment is successful when it induces the rate of viral degradation to proceed more rapidly than that of synthesis. It therefore seems logical to determine whether the amenability of viruses to heat treatment is correlated with their ability to multiply at high temperature, with their stability at high temperatures, or with both.

(a) <u>virus synthesis at high temperature</u>

The rate at which viruses accumulate in plants proceeds slowly at low temperatures, rises with increasing

temperature up to an optimum which is generally within the range 20-30°C, and then declines at temperatures above this optimum. The temperature above which different plant viruses fail to multiply varies considerably. The common strains of carnation ringspot, cucumber mosaic, tobacco necrosis and pelargonium leaf curl viruses do not multiply detectably at 36°C, and they are all readily inactivated by heat treatment (Kassanis, 1952; 1954; 1955). Bawden (1964^{a}) suggested that heat treatment is likely to be successful with those viruses which fail to multiply in their hosts when placed at 36°C about 24 hours after inoculation, and this is supported by Hitchborn's findings (1956; 1957) that certain strains of cucumber mosaic and tobacco ringspot viruses, which have not been successfully inactivated by heat treatment, do multiply at 36°C. However, some viruses which are not amenable to heat treatment, such as potato virus X and clover yellow vein virus, also fail to multiply at 36°C (Closen 1964; Hollings & Nariani, 1966), and some strains of tobacco mosaic, which are extremely heat stable, multiply and accumulate very slowly at 36-38°C (Kassanis, 1957b; Steere, 1952). On the other hand, carnation mottle virus multiplies at 36°C (Kassanis, 1955) but has been completely inactivated in carnation plants by heat treatment (Brierley, 1964). Therefore, it is not possible to account for differences between viruses in susceptibility to heat treatment solely on the basis of their ability to multiply at high temperature.

The reasons why many plant viruses fail to multiply at high temperature is unknown. It is tempting to speculate

that it reflects the hosts' inability to synthesise protein at supra-optimal temperature as viruses rely on much of the hosts' protein synthesising machinery for their replication. For example, the failure of many microorganisms to grow at temperatures slightly above their optimum for growth is correlated with cessation of protein synthesis which is due to the thermolability of their ribosomes (Schiebel et al., 1969) and messenger RNA (Grinsted, 1969). However, cessation of viral synthesis at high temperature can not be accounted for solely in these terms because heat treatment of virus infected plants may result in the production or selection of strains which can multiply readily at high temperature. This phenomenon was first observed by Johnson (1926). These strains generally induce only mild symptoms in their hosts despite reaching high concentrations and, therefore, they have been termed "attenuated" strains.

Kassanis (1957b), in support of the earlier work of Holmes (1934) and Johnson (1947), provided conclusive evidence to show that thermophilic strains of tobacco mosaic virus arise by mutation during heat treatment and not by temperature selection from a pre-existing mixed strain population. Portion of Table 1 from the paper of Kassanis is set out in Table 1 as it succinctly illustrates the method he used, and demonstrates several important points with respect to heat treatment. The two plants in experiment no. 1 were infected with inoculum from the same single lesion. Following treatment, extracts from systemically infected leaves of the plants were inoculated onto Nicotiana glutinosa and ten

TABLE 1.

Extract of data from Table 1 of the paper of Kassanis (1957b).

Experiment No.	Treatment	Treated Plant and its Source	Segregation of Strains into 10 Single Lesions	
			distorting (D)	mild (M)
1	36°C/10 days	plant infected from single D lesion	7	3
	20°C/10 days	plant infected from single D lesion	10	0
2	36°C/19 days	plant D from expt. 1	6	4
3	20°C/7 days	plant M from expt. 2	0	10
	36°C/7 days	plant D from expt. 2	9	1
4	36°C/21 days	plant D from expt. 3	1	7
5	36°C/39 days	plant D from expt. 4	2	8

lesions from each of these were then inoculated back to systemic host plants to provide the information in the last two columns. On the assumption that lesions arise by infection with a single virus particle, for which there appears to be irrefutable evidence (Furumoto & Mickey, 1967a; 1967b), the results indicate that thermophilic strains arise by mutation during heat treatment. They also show that the chance of obtaining these strains increases with the length of heat treatment, and that they do not readily revert to distorting strains when cultured at normal temperatures.

Hollings & Stone (1962) reported the development of thermophilic strains of carnation mottle virus during heat treatment which prevented them from successfully inactivating that virus, and Hollings (1964) had a similar experience with tomato aspermy virus in chrysanthemum.

The long delay in resurgence of symptoms in plants following unsuccessful heat treatment may indicate that the development of thermophilic strains is more common than supposed if the resurgence is due to the reversion of attenuated to normal strains. For example, Hollings (1961) noted redevelopment of symptoms of hydrangea ringspot in hydrangea four months after treatment.

Posnette & Cropley (1958) found that Royal Sovereign strawberries infected with strawberry yellow edge virus seemed healthy for more than one year following treatment before symptoms reappeared, and I have noted (unpublished data) that apple trees appeared healthy for more than two years following heat treatment before it was established that they had not

been freed of apple chlorotic leafspot virus. These times are far greater than the normal incubation period of viruses. An alternative explanation might be that heat treatment results in the stimulated production of some factor by the host, such as the systemic acquired resistance factor studied by Ross (1966), which limits virus multiplication following return of the plants to normal temperature.

The data of Welsh & Nyland (1965), who found that virus-free material could be propagated more often from lateral buds taken after shorter rather than longer periods of treatment, may be explained by postulating the development of thermophilic strains in the roots of their heat-treated apple trees followed by mobilisation of these strains to above-ground parts. As pointed out by Kassanis (1957b), it is not necessary to suppose that all variants which develop at high temperature should be attenuated; rather the methods used for isolating new variants have allowed only attenuated forms to be selected.

On account of attenuation, therefore, it seems unwise to follow Fulton's (1954) suggestion of gradually acclimatising plants to high temperature prior to heat treatment for, although this procedure may result in the heat adaptation of several plant enzymes (Langridge, 1963) and of whole plants or plant parts (Schroeder, 1963; Yarwood, 1961), there are recorded instances of heat adaptation of plant viruses (Joshi & Holmes, 1968; Yarwood & Holm, 1962).

There has been no study of the reason(s) why thermophilic strains, but not the parent forms, of viruses

multiply readily at high temperature. An investigation of this point could provide valuable information on the mechanism of virus therapy by heat treatment. Similarly, there is no information on whether the ease of therapy of different viruses by heat treatment is inversely correlated with their propensity to mutate to thermophilic forms at high temperature.

(b) virus degradation at high temperature

The kinetics of thermal inactivation of plant viruses 'in vitro' was studied by Price (1940, 1933) and Lauffer & Price (1940). They concluded that the inactivation was a first order reaction, which means that the number of particles inactivated at any instant is directly proportional to the number of infective particles present at that time, because irrespective of the number of particles present, the same proportion will always possess energy in the range beyond the energy of inactivation. This implies a direct effect of temperature upon the virus. Different viruses varied in their energies of inactivation.

Ginoza (1958) investigated the kinetics of the thermal inactivation of the RNA from tobacco mosaic virus. The reaction was found also to be one of the first order, and the heat of inactivation was about 13% of that for whole virus particles. This value, of 19,000 cals/mole, was close to that required to hydrolyse a single sugar-phosphate bond in the RNA molecule, which is significant in the light of Gierer's (1957) evidence that a single break anywhere along the length of the RNA molecule was sufficient for inactivation.

The additional energy required for the inactivation of intact virus particles seems to be necessary to break hydrogen bond linkages between the RNA and protein coat so that portions of the RNA become free enough for scission to take place. For instance, Pollard & Dimond (1952) found that the heat of inactivation of tobacco mosaic virus in dried leaf tissue, where hydrogen bond forces would not be operating, was not very different from that obtained for RNA alone. The need to destroy bonds between the RNA and protein coat may also explain why Price (1940) found that the heat of inactivation varied up to six-fold dependent on the pH of the solution. This result may be due to variation in the degree of protonation of viral constituents.

The above conclusions on the kinetics of the thermal inactivation of plant viruses are open to question because infectivity assays were done assuming a linear relationship between local lesion number and virus concentration with a positive slope of unity. This is not a valid assumption, as was emphasised strongly by Bawden (1964$^{a}_{\wedge}$), and one must conclude that these reports do not unequivocally establish the kinetics of thermal inactivation of plant viruses.

The exception to this is a recent report by Babos & Kassanis (1963a) on the thermal inactivation of tobacco necrosis virus 'in vitro'. They found that loss of infectivity was not uniformly exponential. They explained this result by postulating that the virus behaved as if it was a mixture of two components which were inactivated at different rates and that the relative proportion of

components depended on the temperature of heating. In addition, they noted variations between the rate of inactivation of RNA isolated from different strains of the virus; and this they considered to indicate that it is not the cleavage of phosphodiester bonds which causes inactivation. I find these results difficult to interpret. However, an associated investigation on the thermal inactivation of the stable tobacco mosaic virus indicated a first-order reaction was operating.

A common method used to assist in identifying unknown plant viruses which are sap transmissable is to determine their thermal inactivation point (TIP), i.e. the temperature at which no infectivity can be detected after heating inocula 'in vitro' for ten minutes. The TIP of plant viruses vary between about 40^{o}C and 95^{o}C.

However, it is doubtful whether the recorded TIP of plant viruses truly reflect their intrinsic thermal stabilities. The TIP and stability of many plant viruses is much greater in purified preparations than in expressed sap. For example, Francki et al. (1966) found that the TIP of a strain of cucumber mosaic virus in expressed sap was 55^{o}C, while in purified preparations the figure was above 80^{o}C. Even such a stable virus as that causing tobacco mosaic is more heat resistant in purified preparations than in expressed sap (Lauffer & Price, 1940; Price, 1940). However, these differences may be more apparent than real, due to different starting concentrations of virus in the two systems. Gibbs (1969) has suggested that the large number of viruses with a

TIP in the range 50-70°C may not reflect their thermal stability so much as their ability to be adsorbed on the large number of plant proteins which coagulate in sap at this temperature.

The TIP of tomato spotted wilt virus is increased considerably by adding sodium sulphite to the inoculum (Norris, 1946) and increased or decreased by bubbling nitrogen or oxygen, respectively, through the inoculum (Best, 1937). These observations suggest that inactivation was at least partially due to oxidation rather than a direct effect of temperature 'per se'.

Therefore, it is not surprising to find that no relationship exists between the TIP of many plant viruses and their amenability to heat treatment. For example, tomato spotted wilt and tobacco necrosis viruses, both eliminated by heat treatment, have TIP of about 42°C and 92°C respectively, while potato virus Y and tobacco mosaic virus, which are not amenable to heat treatment, have TIP of about 55°C and 95°C respectively. Kassanis (1957a) speculated that these anomalies between behaviour 'in vivo' and 'in vitro' might be due to the viruses which are amenable to heat treatment therapy having low temperature coefficients of heat inactivation. However, there is no published information on this point.

A mechanism by which turnip yellow mosaic virus is inactivated when heated 'in vitro' was revealed by Lyttleton & Matthews (1958). They found that the RNA was released from the virus by heating at about 40°C at pH7. The RNA had a low molecular weight, presumably due to nuclease degradation

following release, because the RNA can be recovered intact (28 s) if precautions are taken to inhibit RNase activity whilst heating (Hitchborn, 1968). A later report (Matthews & Lyttleton, 1959) indicated that RNA was not released from within the virus when heated 'in vivo'. However, the temperature of treatment in their 'in vivo' experiments was only 33°C, and extrapolation of the data from their 'in vitro' tests, suggests that little RNA would be released at this temperature.

This mechanism does not apply universally, or even to all spherical viruses. The RNA in brome mosaic virus does disintegrate at temperatures near 36°C (Kassanis & Lebeurier, 1969) and this virus is amenable to heat treatment (Chiu & Sill, 1963) despite the fact that it multiplies at 36°C (Kassanis & Lebeurier, 1969). However, there is no evidence for the release of RNA from tobacco necrosis virus (Babos & Kassanis, 1963a), and tomato bushy stunt virus (Kassanis & Lebeurier, 1969) although they are readily amenable to heat treatment.

Although the RNA apparently is not released from many spherical viruses cured by heat treatment, their specific infectivity does decline during heating, i.e. the rate of loss of infectivity is much greater than the rate of loss of serological titre or disappearance of particle numbers. This phenomenon has been noted with alfalfa mosaic (Kuhn & Bancroft, (1961), cowpea chlorotic mottle (Kuhn, 1965), bean pod mottle (Gillaspie & Bancroft, 1965), broad bean mottle (Kodama & Bancroft, 1964), tomato bushy stunt (Kassanis, 1952), pea

enation mosaic (Izadpanak & Shepherd, 1966) and tobacco necrosis (Bawden, 1941) viruses. This suggests that heating causes subtle changes in the protein coat which results either in the direct cleavage of RNA attached to it, or allows access by chemicals such as nucleases which disrupt the RNA. Some such explanation seems necessary because nucleic acids are intrinsically stable at the temperatures employed for heat treatment. The latter mechanism (nuclease degradation) is favoured by the results of Harrison (1956) which showed that the rate of inactivation of tobacco necrosis virus at 36°C was greater 'in vivo' than 'in vitro' implying that some factor(s) are produced by the host in response to high temperature which are at least partially responsible for destruction of infectivity.

A study of the kinetics of inactivation during heat treatment 'in vivo' would be valuable in determining whether or not loss of infectivity is a direct effect of temperature on the virus. Unfortunately, this information is not available. The only studies on this subject are of the type illustrated here by some results from the paper of Posnette & Cropley (1958), which set out the proportion of healthy plants obtained after varying periods of treatment. They found that holding Huxley's Giant strawberry plants infected with strawberry mottle virus at 37°C produced 0/4, 0/5, 6/9, 6/9 and 8/9 healthy plants after 6, 7, 8, 9 and 10 days, respectively. This type of data gives no indication of the mechanism of inactivation apart from establishing that it is not an 'all or none' process. However, this information has

great practical importance as it indicates that the chance of successful heat treatment will be greater if the initial virus concentration in the plant is low. Hunter et al., (1959) modified the method of Posnette & Cropley (1956) to take advantage of this situation. They implanted single buds from apple trees infected with the virus causing apple mosaic into virus-free seedlings and heat-treated the budded seedlings shortly after their propagation.

Rich (1969) also used this concept to free potato virus X from infected tubers, a virus which is not normally amenable to heat treatment (Kassanis, 1954). He implanted eyes of virus infected Green Mountain tubers into tubers of the immune varieties Saco and Tawa, as the sap of these varieties contains a substance which reduces the infectivity of potato virus X in Green Mountain sap (Tsou et al., 1967). This reduced the virus content of the Green Mountain eyes so that they could then be freed of the virus by treatment at $38^{o}C$ for 11 days, whereas this treatment would not eliminate the virus from Green Mountain eyes which had been implanted into healthy Green Mountain tubers.

Further data taken to indicate a host-mediated effect on inactivation during heat treatment is that the ease with which cure of a virus is obtained varies with the variety or species of host plant. For example, Posnette & Cropley (1958) had greater difficulty in freeing Fragaria vesca from virus than with the commercial strawberry varieties (F. grandiflora). This result may merely reflect different initial virus concentrations in the cultivars

treated, as F. vesca is a strawberry virus indicator, and virus concentration is normally correlated with symptom severity (Cheo & Pound, 1952; Pound, 1949). However, several other viruses were shown by Kassanis (1957a), Pound (1949) and Hildebrand (1964) to be eliminated at different rates in various hosts, and, in these instances, an explanation in terms of differences in the extent of high temperature induced metabolic responses seems more plausible. Better evidence for this hypothesis is that the rate of virus inactivation is greater in growing plants than in dormant material such as seeds (e.g. Megahed & Moore, 1969; Broadbent, 1965).

V CLASSIFICATION ACCORDING TO BEHAVIOUR DURING HEAT TREATMENT

Many points discussed so far infer that successful therapy by heat treatment cannot be attributed to a direct effect of temperature on the virus particles, but rather that host-induced metabolic changes are at least partially responsible for the observed effects. I have attempted to classify viruses according to their behaviour during heat treatment to see whether this is correlated with one or more characteristics which might explain the manner by which host-mediated inactivation proceeds. Particle shape was used as an additional criterion in the classification. The reviews by Gibbs (1969) and Hollings (1965a) proved valuable during this work, and the virus names used are those listed by Martyn (1968).

The groupings are somewhat subjective for three

reasons. Firstly, there are recorded successes following heat treatment which are obviously incorrect and which have not been verified. For example, Thomson (1958) reported freeing potato tubers from potato viruses A, S and Y by holding them in air at 30-38°C for as little as 7 days, whereas other workers have had to resort to prolonged heat treatment combined with tip culture to eliminate these viruses (e.g. Stace-Smith & Mellor, 1968). Brierley (1957) reported obtaining virus-free tip cuttings from plants infected with hydrangea ringspot virus after four weeks at 38°C, but Hollings (1965a) was unable to repeat this and suggested that Brierley's apparent success resulted from testing his material for infection too soon after completion of heat treatment. Secondly, few have attempted to determine the minimum temperature-time combinations necessary to free plants from many viruses. The general policy has been to treat plants at as high a temperature for as long as they will survive. For example, the only information available on the heat treatment of citrus exocortis virus is that shoot tips propagated from infected lemon trees 200-400 days after treatment at 38°C subsequently proved to be healthy (Stubbs, 1968). Thirdly, many people have successfully eliminated viruses by adopting the method of Campbell (1962) whereby small apical tip cuttings are taken from the shoots of plants formed during treatment without having investigated whether larger cuttings, or indeed the whole plants, were free from infection.

In general, viruses are listed as being readily cured if the treatment was carried out at 40°C or less for no longer than four weeks and that, if cuttings were taken from the plants, they were not of the small apical type used by Campbell (1962).

It should be noted that this classification does not agree closely with that of Nyland & Goheen (1969) which was independently constructed at about the same time. However, assortment into groups is somewhat subjective.

(a) readily heat treated, leaf hopper transmitted

disease	reference
aster yellows	Granados & Chapman (1968) Kunkel (1937, 1941)
bayberry yellows	Raychaudhuri (1953)
cherry little cherry	Nyland & Reeves (1962)
chrysanthemum flower distortion	Brierley & Smith (1957)
clover dwarf	Valenta (1962)
clover phyllody	Posnette & Ellenberger (1963) Valenta (1959)
clover wound tumour	Selsky (1960) Sinha (1967)
delphinium yellows	Posnette & Ellenberger (1963)
grapevine flavescence doree	Caudwell (1966)
Guatemala grass spikiness	Mulder (1963)
lucerne witches broom	Kunkel (1952)
mulberry dwarf	Kulkarni (1969) Tahama (1964)
Opuntia witches broom	van der Meer (1967)
parastolbur	Valenta (1962)
peach phony	Hutchins & Rue (1939)
peach rosette	Kunkel (1936)
peach X disease	Hildebrand (1941) Jensen (1968)
peach yellow leaf roll	Nichols & Nyland (1952)
peach yellows	Kunkel (1936)
Pennisetum clandestinum chlorosis and stunting	Bruehl (1953)
potato witches broom	Kunkel (1943) Thomson (1956)
rubus stunt	Thung (1952)
stolbur	Valenta (1959)

disease	reference
sugar cane chlorotic streak	Abbott (1959) Antoine & Ricaud (1964) Bell (1933) Martin (1933) Wiehe (1966)
sugar cane sereh disease	Houtman (1925) Wilbrink (1923)
sugar cane white leaf	Ling & Chuang-Yang (1965) Liu (1963) et al.
Vaccinium (cranberry) false-blossom	Kunkel (1945)
Vaccinium (blueberry) stunt	Nyland & Goheen (1969)

This group of diseases are all readily inactivated by heat treatment, using either air or water treatments, or both. They are transmitted by leaf-hoppers in which the infective agents multiply. Heat treatment therapy has been successful both on infected plants and infective vectors. Mycoplasmas have been implicated as the infective agent in many of these diseases during the last three years. These include aster yellows, clover dwarf, clover phyllody, lucerne witches broom, porustolbur, potato witches broom and stolbur. Some of the diseases have been cured by treatment with members of the tetracyclines group of antibiotics. However, no one has yet successfully isolated any of these organisms and reinoculated them back into healthy plants to induce disease.

Clover wound tumour results from infection with a large spherical virus, 70 nm in diameter, and might have been classified more correctly in group (b).

Crimean yellows is the only leaf-hopper transmitted disease which has not responded to heat treatment (Valenta, 1959), but the treatment was only for 6 days at 43.5°C.

(b) readily heat treated, known particle shape

virus	disease	reference
abutilon variegation	abutilon variegation	Kassanis (1954)
alfalfa mosaic	alfalfa mosaic	Frosheiser (1969)
arabis mosaic	cucumber stunt	Hollings (1963b)
	grapevine fan leaf	Ducreux (1963)
		Galzy (1966)
		Woodham (1967)
	hop nettlehead mottle strain	Glazewska (1963)
		Hollings (1963b)
broad bean mottle	broad bean mottle	Hollings & Stone (1965b)
brome mosaic	bromegrass mosaic	Chiu & Sill (1963)
carnation etched ring	carnation etched ring	Brierley (1964)
carnation mottle	carnation mottle	Brierley (1964)
		van Os (1964)
		Paludan (1964)
		Paludan (1965)
carnation ring spot	carnation ring spot	Brierley (1964)
		Hollings (1961)
		Hollings & Stone (1965a)
		Kassanis (1954)
		van Os (1964)
citrus exocortis	citrus exocortis	Stubbs (1968)
citrus psorosis	infectious variegation psorosis	Majorana (1966)
		Grant (1957)
cucumber mosaic	cucumber mosaic	Hitchborn (1956)
		Kassanis (1954)
	passion fruit woodiness	Taylor (1959)
cymbidium ring spot	white clover virus disease	Hollings & Stone (1965b)
pelargonium leaf curl	carnation Italian ring spot	Hollings (1963a)
		Hollings & Stone (1965b)
	pelargonium leaf curl	Hollings (1962a)
	tomato bushy stunt	Kassanis (1954)
potato leaf roll	potato leaf roll	Fernow et al. (1962)
		Hamid & Locke (1961)
		Kassanis (1950)
		Nagaich (1963)
		Nagaich & Upreti (1964)
		Sip (1965)
prune dwarf	peach stunt	Nyland (1960)
	prune dwarf	Ehlers (1957)
		Megahed & Moore (1969)
		Nyland (1960)

virus	disease	reference
prune dwarf	sour cherry yellows	Ehlers (1957) Nyland (1960)
Prunus necrotic ring spot	almond mosaic	Majorana & Martelli (1966)
	apple mosaic	Hunter et al. (1959) Kegler (1959) Nagaich (1963) Posnette & Cropley (1956) Thomsen (1968)
	cherry ring spot	Ehlers (1957) Megahed & Moore (1969) Nyland (1960)
	hop nettlehead	Glazewska (1963)
	plum line pattern	Ellenberger (1960)
	rose mosaic	Holmes (1960) Traylor et al. (1967)
satellite virus	satellite virus infection	Rees et al. (1970)
sugar cane ratoon stunting	ratoon stunt	El-Banna et al.(1967) Lee & Liu (1961) Liu et al. (1963) Singh (1967) Steib & Forbes (1958) Steindl & Hughes (1953) Wiehe (1966)
tobacco necrosis	tobacco necrosis	Babos & Kassanis (1963b)
tobacco ring spot	anenome necrosis	Hollings (1965c)
	tobacco ring spot	Hitchborn (1957)
tomato aspermy	chrysanthemum aspermy	Fenton (1969) Kristensen & Thomsen (1958) Kassanis (1954)
tomato black ring	celery yellow vein	Hollings (1965d)
tomato ring spot	grapevine yellow vein	Nyland & Goheen (1969)
	peach yellow bud mosaic	Nyland & Goheen (1969)
tomato spotted wilt	tomato spotted wilt	Bald (1970) Hollings (1962b)
turnip crinkle	turnip crinkle	Hollings & Stone (1963) Hollings & Stone (1965b)

This list cuts across the groupings in the system of classification proposed by Gibbs (1969), which relies greatly

on mode of transmission. The viruses listed here are transmitted in such diverse ways as by sap, seed, pollen, insects, nematodes and fungi. However, there are several properties common to many, if not all, members of this group which may explain why they are so readily amenable to heat treatment.

Most are spherical in shape, generally about 25-35 nm in diameter. Satellite virus is somewhat smaller. Alfalfa mosaic has bullet-shaped particles, but Gibbs (personal communication) has suggested it may be closely related to these viruses because of similarities in chemical composition and on the evidence of Bancroft et al. (1967) that reassembly of the components of cowpea chlorotic mottle virus, which is normally spherical, can lead to the formation of particles closely resembling those of alfalfa mosaic under certain conditions. It may be that a similar situation applies to the large, complex and spherical tomato spotted wilt virus.

These viruses are all ribonucleoproteins and contain a high proportion of single-stranded RNA. The guanine plus cytosine content of their RNA is generally low (see Gibbs, 1969). This condition is associated with mesophily in bacterial systems (Stenesh & Holazo, 1967; Zeikus et al., 1970) and is thought to be due to the more limited capacity for hydrogen bonding within such RNA molecules.

The high RNA content, generally about 20% (Gibbs, (1969), may result in this infective material being less well

protected by their protein coats than most other viruses. Support for this comes from the findings that several of these viruses appear to be inactivated by ribonuclease, viz. alfalfa mosaic (Bol & Veldstra, 1969), broad bean mottle (Kodama & Bancroft, 1964), brome mosaic (Incardona & Kaesberg, 1964), citrus exocortis (Semanick & Weathers, 1968), cucumber mosaic (Francki, 1968), pelargonium leaf curl (Dorne, 1968), Prunus necrotic ring spot (Diener & Weaver, 1959), tomato spotted wilt (Bald, 1964) and tobacco necrosis (Babos & Kassanis, 1962) viruses. Two other closely related viruses likely to be amenable to heat treatment, cowpea chlorotic mottle and turnip yellow mosaic viruses, also are inactivated by ribonuclease (Bancroft et al., 1967; Kaper & Jenifer, 1968). The RNA of tobacco ring spot virus also may be degraded within intact virus particles, but in this case the reaction appears to be non-enzymic (Schneider & Diener, 1968).

It is not absolutely certain in all these instances whether the apparent inactivation was not at least partially due to a competitive effect between the virus and enzyme for infectible sites during the assay procedure. Loring (1942) found that ribonuclease strongly inhibits the infectivity of tobacco mosaic virus in this way.

Further support for inactivation by ribonuclease comes from the findings that some of these viruses contain components with differing proportions of RNA (see Gibbs, 1969; Hollings & Stone, 1965b). In addition, the infectivity of cucumber mosaic and tobacco necrosis viruses

is almost as great, or greater, in phenol than in water extracts of diseased leaves (Schlegel, 1960).

It is of interest that the ribonuclease concentration increases markedly in plants under various conditions of stress such as senescence (Wyen et al, 1969), mechanical injury (Shinde et al., 1964), water shortage (Dove, 1967) and parasitic attack (Diener, 1961).

Studies on the properties of many of these viruses have been limited due to difficulties in purification (e.g. see Scott, 1963). One reason for this is that many fail to reach high concentrations in their hosts (Bawden, 1964) and that specific infectivity declines rapidly after reaching a peak 10-20 days following infection (Steere, 1959). Purification from naturally infected hosts may be particularly difficult as their virus concentration only reaches high levels during the cooler winter months (e.g. Oertel, 1967) and symptoms tend to become heat-masked during the summer concomitant with a decline in infectivity (see Smith, 1957). The mechanism responsible for this 'in vivo' decline in specific infectivity is unknown, although several possible explanations have been advanced suggesting virus induced increases in the production of one or more 'in vivo' virus inhibitors by the host (Kuhn & Bancroft, 1961).

Another difficulty in purification of some of these viruses is that high molarity buffers must be used to extract them from plant tissues (e.g. Grogan et al., 1963), as their protein structure may be altered by slight changes in pH

(Bancroft et al., 1967; Incardona & Kaesberg, 1964) and because plant sap extracts are, themselves, strongly buffered at pH values not optimal for viral stability. However, this introduces an enigma because many of these viruses are unstable in solutions of high ionic strength (Gibbs, 1969; Scott, 1963).

Several of these, and related, viruses are also unstable during purification because they are inactivated by oxidised phenolic compounds. These include alfalfa mosaic (Saksena & Mink, 1970), cucumber mosaic (Harrison & Pierpoint, 1963), prune dwarf (Hampton & Fulton, 1961), Prunus necrotic ring spot (Hampton & Fulton, 1961), sowbane mosaic (Saksena & Mink, 1970), tobacco streak (Fulton, 1949), tomato spotted wilt (Bald & Samuel, 1934) and Tulare apple mosaic (Mink, 1965) viruses.

An 'in vivo' role also may occur with this inactivating system as the concentration of polyphenoloxidases and their substrates often increases in damaged, stressed, and virus infected plant tissues (Goodman et al., 1967), and Hampton & Fulton (1961) found that the infectivity of Prunus necrotic ring spot virus increased in plants sprayed with an 'in vivo' inhibitor of polyphenoloxidase.

There is insufficient information about the amino acid sequence and content of plant viruses to explain how these viruses become inactivated by oxidised phenolic compounds. However, it is of interest that tobacco mosaic virus, which is not affected by oxidised phenolic compounds

(Saksena & Mink, 1970), contains only one cysteine residue per subunit (Knight, 1964).

(c) readily heat treated, unknown particle shape

virus	disease	reference
apple rubbery wood	apple rubbery wood	Posnette et al. (1962) Welsh & Nyland (1965)
black currant reversion	black currant reversion	Campbell (1965)
cassava mosaic	cassava mosaic	Chant (1959)
cherry necrotic rusty mottle	cherry necrotic rusty mottle	Nyland (1959)
chrysanthemum green flower	chrysanthemum green flower	Hollings (1963a)
chrysanthemum ring spot	chrysanthemum ring spot	Hollings & Kassanis (1957)
chrysanthemum stunt	chrysanthemum stunt	Hollings & Kassanis (1957)
citrus stubborn disease	citrus greening	McLean et al. (1968)
	citrus stubborn	Calavan (1968) Olson & Rogers (1969)
	citrus yellow shoot	Lin (1964) Lin & Lo (1965)
fig mosaic	fig mosaic	Casalicchio (1964)
Hibiscus leaf curl	Hibiscus leaf curl	Mukherjee & Raychaudhuri (1966)
peach (Muir) dwarf	peach (Muir) dwarf	Nyland (1960)
pear bark necrosis	pear bark necrosis	Posnette et al. (1962)
pear stony pit	pear stony pit	Christoff (1958)
pear vein yellows	pear vein yellows	Posnette et al. (1962)
plum bark split	plum bark split	Ellenberger (1960)
raspberry (black) necrosis	component of raspberry mosaic	Converse (1966)
raspberry leaf mottle	component of raspberry mosaic	Chambers (1954) Chambers (1961)
raspberry leaf spot	component of raspberry mosaic	Chambers (1954)
strawberry crinkle	strawberry crinkle	Mellor & Fitzpatrick (1961)

virus	disease	reference
strawberry crinkle	strawberry crinkle	Miller (1954) Posnette (1953) Posnette & Cropley (1958) Posnette et al. (1953) Posnette & Jha (1960)
strawberry mild yellow edge	strawberry mild yellow edge	Miller (1954) Posnette & Cropley (1958)
strawberry mottle	mottle	Mellor & Fitzpatrick (1961) Posnette & Cropley (1958)
	leaf tattering	Fitzpatrick et al. (1954)
strawberry witches broom	strawberry witches broom	Mellor & Fitzpatrick (1961)

These viruses, readily amenable to heat treatment, are transmitted in a variety of ways. A few are transmitted only by vegetative propagation, and some others spread naturally, although their vectors are unknown.

None are transmitted mechanically and, therefore, there is no information on their inherent properties. As further information on their characteristics becomes known, it will be interesting to see whether they may be included in either group (a) or (b) discussed previously. Assumption that they are related to the viruses in group (b) might aid in devising methods for their mechanical transmission and purification.

(d) not readily heat treated, known particle shape

virus	reference
apple chlorotic leaf spot	Campbell (1962)
apple stem pitting	Campbell (1962)
carnation latent	Brierley (1964)

virus	reference
carnation vein mottle	Quak (1961)
chrysanthemum virus B	Hakkaart & Quak (1964)
citrus tristeza	Desjardins et al. (1959)
hydrangea ring spot	Brierley (1957)
plum pox	Kegler (1967)
poplar mosaic	Berg (1964)
potato virus A	Thomson (1958)
potato virus S	Mellor & Stace-Smith (1970)
potato virus X	Mellor & Stace-Smith (1970)
potato virus Y	Thomson (1958)
sweet potato internal cork	Hildebrand (1964)
turnip mosaic	Holmes (1965)

This list is not a complete bibliography for each virus. One reference only is given in each case to indicate that plants infected with these viruses are only cured by prolonged heat treatment, normally in combination with meristem tip culture. In a few instances, heat treatment in conjunction with the use of chemicals, such as malachite green, has been successfully employed.

All of these viruses are rod-shaped, and the particles have much lower proportions of RNA:protein than those in group (b). This suggests that the RNA is much better protected in these particles. None of them are known to be inactivated by ribonuclease and, when heated 'in vitro', the rate of loss of infectivity is paralleled by the rate of loss of antigenicity (e.g. Close, 1964).

In addition, these viruses reach high concentrations in their host plants (e.g. Sampson & Taylor, 1968) and specific infectivity does not decline markedly after a peak concentration is reached. Diseases induced by these viruses do not become heat masked during the warmer summer months. None are known to be inactivated by oxidised phenolic

compounds, although they may be precipitated by tannins. Generally, they are stable in solutions over a wide range of pH and ionic strength.

All of these properties are in direct contrast to those viruses placed in group (b) and may explain the difficulty which various workers have had in obtaining healthy material from plants infected with them by heat treatment.

(e) not readily heat treated, unknown particle shape

virus	reference
apple leaf pucker	Welsh & Nyland (1965)
apple (Malus) platycarpa dwarf	Campbell (1962)
apple (Malus) platycarpa scaly bark	Campbell (1962)
apple Spy 227 epinasty & decline	Welsh & Nyland (1965)
cherry (sour) green ring mottle	Nyland & Goheen (1969)
chrysanthemum rosette	Brierley & Smith (1958)
gooseberry vein banding	Jones & Vine (1968)
grapevine asteroid mosaic	Nyland & Goheen (1969)
grapevine corky bark	Nyland & Goheen (1969)
grapevine leaf roll	Nyland & Goheen (1969)
peach stubby twig	Nyland & Goheen (1969)
strawberry latent C	Bolton (1967)
strawberry vein banding	Bolton (1967)

As with the previous group, no attempt has been made to list a complete bibliography on the heat treatment of each of these viruses. A single representative example is given in each instance. All but three of these viruses have no known vectors, and none of them are mechanically transmissable. Their inherent properties are therefore unknown.

(f) not cured by heat treatment

Hollings (1965a) listed several viruses which at that time had not been cured by heat treatment. Since then,

successes have been achieved with some of these, and other viruses have been added to the list of those which cannot be successfully heat treated (Nyland & Goheen, 1969). The properties of these viruses, where they are known, are similar to those in group (d). The exception is potato spindle tuber virus, which is thought to exist as free double-stranded RNA in infected cells (Diener & Raymer, 1967).

With the right combination of time, temperature and technique, there seems to be no reason why any virus infection cannot be cured by heat treatment in combination with other methods. Bald (1971) has recently freed tobacco plants from tobacco mosaic virus by heat treatment in combination with the culture of tip cuttings.

VI CONCLUSIONS

The mechanisms whereby plants are freed from virus infection by heat treatment are unknown. Kassanis & Posnette (1961) have postulated that elevating the temperature differentially alters the relative rates of multiplication and degradation of virus particles.

There is a correlation between the ability of viruses to multiply at high temperature and their amenability to heat treatment, but this is far from perfect. The reason why some viruses cease to multiply above a certain temperature is unknown.

The evidence that inactivation of viruses being heated is a first order reaction is unconvincing. Rather,

many observations suggest that heat induced changes in host metabolism at least are partially responsible for the inactivation. However, this matter has not been investigated. Grouping of viruses which are successfully and readily heat treated reveals that several of these have many properties in common which might explain the mechanism of 'in vivo' inactivation.

The work described in this thesis was designed in an attempt to resolve some of these questions using tomato aspermy virus, which is readily amenable to heat treatment, as the test virus.

EXPERIMENTAL DETAILS AND RESULTS.

I PLANT CULTURE

In most instances, individual systemic and local lesion host plants of the viruses used in this work were grown in 3" plastic pots containing UC Mix I(c) and watered either once or twice weekly, dependent on size, with liquid fertiliser L7 (Baker, 1957). Occasionally, experiments required that plants be grown in solution culture. The basic components of the solutions were those listed by Hewitt (1966) under the modified Long Ashton formula. The solutions were renewed at weekly intervals and were connected to a compressor timed to aerate them for 10 minutes every half hour.

In a few cases, virus was cultured in excised leaves. These were floated on various types of solutions contained in 9 cm x 9 cm shallow plastic dishes in a room with temperature controlled at 21°C and illuminated with a combination of fluorescent and incandescent lights to give an intensity of 1,200 foot candles at the leaf surface.

Heat treatment of plants was done in a C.S.I.R.O. type BD phytotron cabinet (Plate 1) similar to that used by Stubbs (1963b). The cabinet was run continuously at 36°C. To aid plant survival, pots were placed in galvanised trays filled with coarse sand to maintain an even water supply by capillarity to the plants. As well, water was sprayed continuously through a fine nozzle, which delivered 1½ gallons/hour, into the air circulation chamber so that relative humidity within the cabinet was kept at about 70%.

PLATE 1.

C.S.I.R.O. type BD phytotron cabinet showing plants on galvanised trays filled with sand for watering by capillarity and spray hose in dorsal duct to maintain humidity.

Either an open-ventilated or air-conditioned, insect-proof glasshouse served as a control environment for comparison with plants grown in the cabinet. The mean monthly minimum and maximum temperatures recorded in the open glasshouse are listed in Appendix 2. The other glasshouse, which became available during the final year of the project, maintained temperature between 13 and 24°C and relative humidity at close to 60% (Plate 2). These facilities did not permit replication of environments. However, replication was possible within environments, and statistical comparisons between them were considered valid if their separate error variances were homogeneous (Snedecor, 1956). It was rare for this condition not to be satisfied.

II VIRUS ISOLATES AND THEIR CHARACTERISATION

(a) selection of TAV

Tomato aspermy virus was found to be infecting 99 out of 110 different chrysanthemum plants selected at random from eight sites in the Hobart district. The virus, previously unrecorded in Tasmania (Wade et al., 1959), was detected by mechanical inoculation to *Chenopodium amaranticolor*, *Nicotiana glutinosa*, *Nicotiana tabacum* and *Petunia hybrida*. All of the aspermy-infected chrysanthemums were then tested for the presence of other possible contaminating latent viruses in both of two different ways - firstly by examining sap extracts from the plants, negatively stained with 2% neutral phosphotungstic acid, under the

PLATE 2.

Portion of air-conditioned, insect-proof glasshouse showing tobacco plants growing in solution culture in foreground, C. amaranticolor in middle-ground, and N. tabacum in background.

electron microscope (Sampson & Taylor, 1968) and secondly, by stub-grafting shoots from the plants into the chrysanthemum indicator varieties "Goodnews" and "Mistletoe" (Brierley, 1955).

The aspermy isolate used in this study (TAV), which originated from a chrysanthemum plant growing in the Royal Hobart Botanic Garden, was selected because it was one of the few not contaminated with other viruses and because it induced severe symptoms in tobacco.

(b) host range of TAV

In those plants which were hosts of TAV, symptoms developed on inoculated leaves within 5-10 days and, when observed, on systemically infected leaves within 10-15 days. However, these times were not diagnostic as they varied according to the concentration of infective particles in the inoculum. Brief descriptions of these symptoms are set out below (species were recorded as immune when several attempts to recover TAV from plants which did not develop symptoms following inoculation failed):

* Brassica pekinensis: immune.

Chenopodium amaranticolor: light brown local lesions less than 1 mm in diameter; red coloured halos around lesions on younger leaves (Plate 3); no systemic infection.

* Chenopodium quinoa: white-yellow local lesions 1-2 mm in

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* new records of hosts and non-hosts of tomato aspermy virus.

PLATE 3.

Local lesions induced on opposite half leaves of C. amaranticolor by inocula containing different concentrations of TAV.

diameter on inoculated leaves; no systemic infection.

Cucumus sativus: sporadic chlorotic local lesions 2-4 mm in diameter on cotyledons; no systemic infection.

* Cucurbita maxima: immune.

Datura stramonium: immune.

Gomphrena globosa: immune.

Lycopersicon esculentum: light and dark green mottle on inoculated and systemically infected leaves together with development towards a "fern leaf" appearance and the formation of enations.

Nicotiana tabacum var. Hickory Prior: light green chlorotic spots and ringspots 1-3 mm in diameter on inoculated leaves sometimes coalescing to form line patterns and becoming necrotic when concentrated inocula were used; these symptoms also developed on systemically infected leaves as well as interveinal raised areas of darker green tissue; leaves tended to a filiform shape and to develop enations on their under sides (Plate 4).

: other varieties and species of tobacco reacted similarly except for N. glutinosa which developed large leaf enations and a pronounced "fern leaf" appearance (Plate 5).

Petunia hybrida: diffuse systemic chlorotic mottle.

* Physalis floribunda: faintly chlorotic local lesions 1-2 mm in diameter on inoculated leaves followed by a diffuse systemic chlorotic mottle.

Pisum sativum: immune.

* Solanum melongena var. esculentum: large sporadic black

PLATE 4.

Symptoms of TAV infection on inoculated leaves of *N. tabacum* var. Hickory Prior showing chlorotic spots, ringspots and line patterns.

PLATE 5.

Symptoms of TAV infection on N. glutinosa showing development of a pronounced "fern leaf" appearance and the formation of leaf enations.

local lesions about 1 cm in diameter on inoculated leaves; no systemic infection.

Tetragonia expansa: faintly chlorotic local lesions 2-4 mm in diameter; no systemic infection.

* Vicia faba: immune.

Vigna sinensis: dark brown local lesions 1-2 mm in diameter on primary leaves; no systemic infection.

Zinnia elegans: chlorotic spots and ringspots up to 2 cm in diameter which occasionally turned necrotic on both inoculated and systemically infected leaves; extensive necrotic vein banding and veinal necrosis; individual flowers were small, emerged unevenly from their buds, and had malformed and faded petals (Plate 6).

The symptoms induced by TAV, described above, were indistinguishable from those caused by a Victorian isolate of the virus (kindly supplied by Mr. R. H. Taylor, Victorian Plant Research Institute). They were also in good agreement with previous descriptions of the symptoms induced by tomato aspermy virus on those plants which are not asterisked in the above list (Blencowe & Caldwell, 1949; Govier, 1957; Grogan et al., 1963; Hollings, 1955; Lawson, 1967; Smith, 1957).

(c) maintenance of TAV

TAV, isolated originally from chrysanthemum, was maintained in N. tabacum var. Hickory Prior. It was transferred at about monthly intervals to young tobaccos using an inoculum derived from several infected plants. The virus maintained by serial passage through tobacco plants

PLATE 6.

Symptoms of TAV infection on *Zinnia elegans* showing veinal necrosis and the development of small flowers with distorted and faded petals.

in the glasshouse was compared, at regular intervals, with aliquots of the original TAV inoculum which had been lyophilised and stored under vacuum at -10°C. As well, possible accidental contamination of TAV in the glasshouse with potato virus X and tobacco mosaic virus was checked frequently by inoculation to G. globosa and N. glutinosa, respectively. These tests indicated that there was no evidence for a change in the strain of TAV maintained in the glasshouse throughout the period of this work, or that TAV ever became contaminated with other viruses.

Unless stated otherwise, all experiments were done with TAV in Hickory Prior.

(d) properties of purified TAV

TAV was purified from tobacco plants, inoculated 14 days previously, by triturating them (1:1, W:V) with 0.3 M sodium phosphate buffer (pH 7.5) containing 0.01 M cysteine hydrochloride. The homogenate, after filtering through cheesecloth, was emulsified with one third volume of chloroform (Schneider, 1953) and the clarified supernatant was then subjected to three cycles of differential centrifugation. The virus was pelleted using a Beckman L2-65 ultracentrifuge fitted with a no. 30 or no. 65 rotor spun at 30,000 r.p.m. for 2 hours or at 50,000 r.p.m. for 1 hour, respectively. The virus was resuspended in 0.1 M sodium phosphate buffer (pH 7.5). All the above operations were carried out at 0°C.

The final preparation was negatively stained with

2% neutral phosphotungstic acid and examined under an electron microscope. Large numbers of approximately spherical particles about 25-30 nm were observed. However, few appeared intact, which may have been due to the staining procedure adopted (Francki et al., 1966), because these particles sedimented uniformly in the analytical ultracentrifuge with an estimated $s_{20,w}$ of about 90. Examination of the preparation with both the electron microscope and analytical ultracentrifuge indicated that it contained a considerable amount of phytoferritin. No other contaminants were apparent (Plate 7).

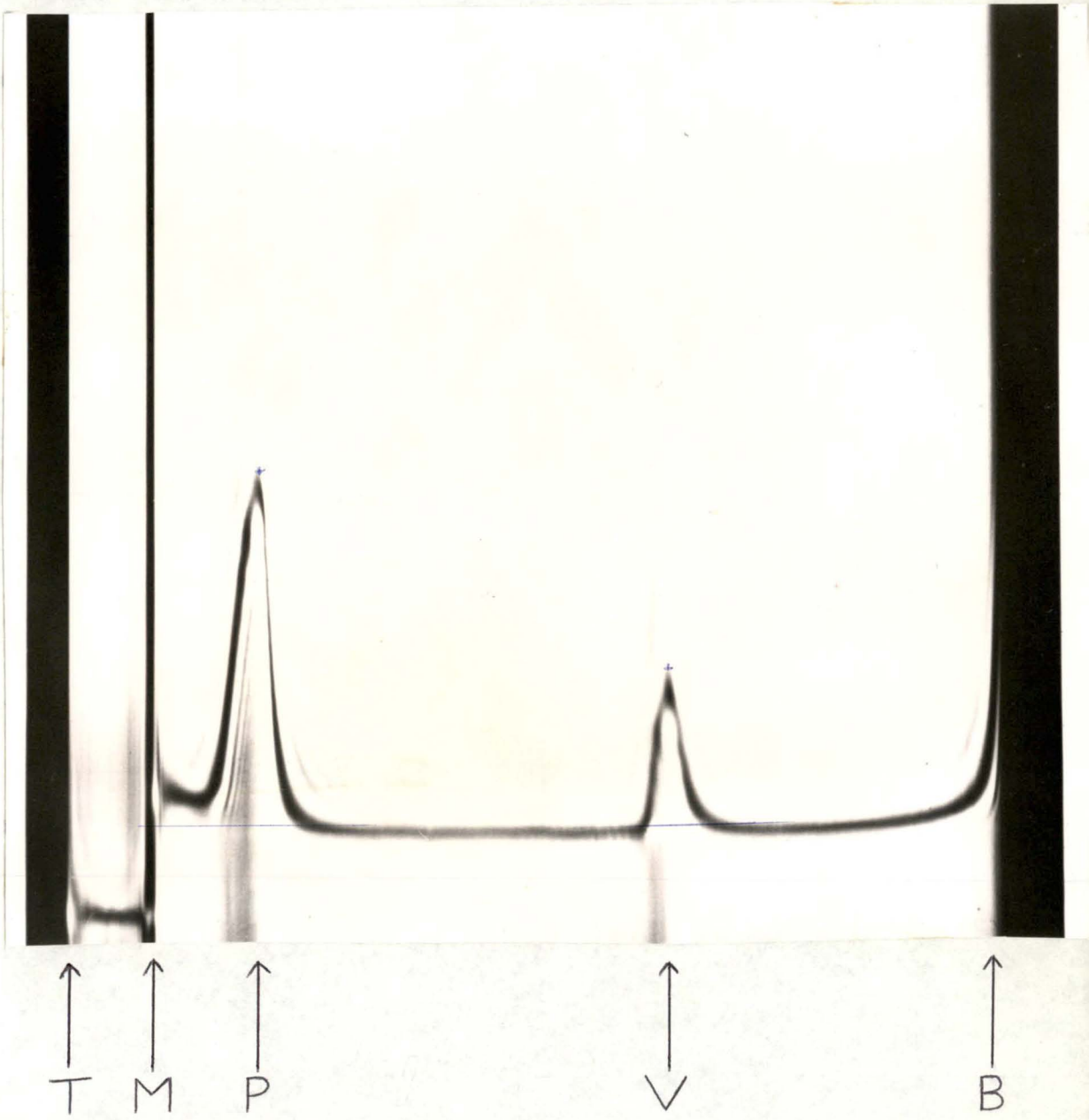
(e) serological properties of TAV

An antiserum to TAV was produced by Fulton's (1968) method of injecting rabbits intramuscularly with 1 ml of purified virus (adjusted to an absorbance of 5.0 at 260 nm) emulsified with 1 ml of Freund's incomplete adjuvant. Injections were repeated ten times at three-daily intervals. The serum was collected three days after the last injection, mixed with an equal volume of glycerol, and stored at $-10^{o}C$. Immunological tests were done in 0.5% agarose gel (containing 0.85% sodium chloride, 0.01% sodium azide and neutral 0.01 M sodium phosphate buffer) in petri dishes by the double diffusion method (Ouchterlony, 1962). Wells were cut in the gel 7 mm in diameter and 5 mm apart. When appropriate, the antiserum was diluted with 0.85% sodium chloride. Results of these tests were on the basis of observations made 48 hours after incubation at $25^{o}C$.

PLATE 7.

Schlieren diagram of a TAV preparation purified from tobacco photographed 16 minutes after reaching speed at 35,600 rpm with a bar angle of 65°. An identical diagram was obtained after heating the preparation at 36°C/1 hour.

(T = top of cell, M = meniscus, P = phytoferritin peak, V = virus peak and B = bottom of cell).


T
M
P
V
B

Tests between extracts from TAV infected plants and its homologous antiserum resulted in the formation of two precipitin lines. One of these appeared close to the antiserum well, and was due to the presence of an antibody in the serum which reacted with a normal host component, presumably phytoferritin. The concentration of this antibody in the serum was very low because this precipitin line never formed when either the antiserum or plant extract was diluted more than two-fold. Apart from this observation, single precipitin lines and reactions of identity occurred in every instance when all possible combinations of TAV and its antiserum, the Victorian isolate of aspermy and its antiserum, and an antiserum prepared against a Californian isolate of aspermy (kindly supplied by Dr. R. H. Grogan, University of California, Davis) were tested against each other. However, TAV did not react with an English isolate of aspermy (kindly supplied by Dr. O. M. Stone, Glasshouse Crops Research Institute, Sussex).

(f) other virus isolates

The U1 strain of tobacco mosaic virus (kindly supplied by Dr. R. I. B. Francki, Waite Institute, Adelaide) was stored in the cold following partial purification from tobacco by Bawden & Pirie's method as outlined by Steere (1959).

III DEVELOPMENT OF ASSAY TECHNIQUE

It seemed essential to develop a very sensitive and accurate assay procedure because tests on heat treated

plants would involve measuring low concentrations of infective TAV particles. This was particularly necessary because I obtained only very weakly infective inocula when using the homogenising medium which Grogan et al. (1963) recommended for the extraction of tomato aspermy virus from tobacco.

Many factors are known to alter the efficiency of mechanical transmission and the susceptibility of host plants to infection (Yarwood, 1957). The results of experiments designed to examine some of these factors in order to increase the sensitivity of assaying TAV infectivity are described below.

(a) choice of assay host

Several experiments were done to compare a range of local lesion hosts of TAV. These established that their relative merits, in terms of decreasing order of sensitivity, were:

Chenopodium amaranticolor

Physalis floribunda

Chenopodium quinoa

Nicotiana glutinosa

Vigna sinensis var. Black, Blackeye

Cucumis sativus var. Long Green

Tetragonia expansa

Lesions on P. floribunda, N. glutinosa and T. expansa were only faintly chlorotic and could be counted accurately only after the leaves had been decolourised in

alcohol and stained with iodine (Plate 8). This was considered to be a serious disadvantage. Cucumber and cowpea plants had the advantage in that their cotyledons and primary leaves, respectively, responded very similarly to inoculation. However, they were far too insensitive for use as assay hosts in this work.

C. amaranticolor was by far the most sensitive, and was therefore the obvious choice to use as a local lesion assay host for TAV. The lesion numbers were often extremely large (> 1000/half leaf), but they could be counted accurately ($\pm$1%) by stabbing them with a needle while examining the leaves at a 16X magnification under a dissecting microscope.

A disadvantage of C. amaranticolor was that there were often very large differences between the response to inoculation of leaves of different physiological age (Table 2). Younger leaves were generally more susceptible, although this was not always the case. Leaves of similar physiological age on apparently similar plants of C. amaranticolor also responded very differently to inoculation (Table 3) in spite of the fact that the plants were decapitated 24 hours previously, a procedure which Youden & Beale (1934) reported to reduce variability in plant response. However, opposite halves of individual leaves reacted similarly to inoculation, the variation between means generally being less than 10%. These findings made it necessary to compare inocula on opposite

PLATE 8.

Local lesions induced by TAV on Tetragonia expansa shown up by decolourisation in alcohol followed by staining with iodine. These lesions were faintly chlorotic and barely visible on untreated leaves. A similar situation applied to the local lesions formed on the inoculated leaves of Physalis floribunda and Nicotiana glutinosa.

TABLE 2.

The response of C. amaranticolor leaves of different physiological age to inoculation with TAV.

Physiological Leaf Age	Lesion No./Leaf*
younger**	1361
intermediate	221
older	15

* each figure represents the mean of 30 leaves; differences between means were highly significant.

** On each plant, the younger leaf was that which was almost fully expanded and which had only a slight reddish tinge on its upper surface; the other two leaves on each plant were those immediately below the younger leaf.

TABLE 3.

The response of C. amaranticolor leaves of similar physiological age to inoculation with TAV among a batch of 16 apparently similar plants.

Plant No.	Lesion No. / Leaf
1	560
2	40
3	281
4	735
5	601
6	306
7	667
8	635
9	419
10	583
11	393
12	221
13	498
14	552
15	374
16	658
Mean	470
Standard Deviation	191

leaf halves. When more than two inocula were to be compared, balanced incomplete block designs had to be employed which allowed estimation of the variation due to 'between plant' and 'between leaf age' responses. The necessity to use plants as replicates meant that normally it was not possible to compare more than 4 or 6 inocula in any one experiment.

(b) additions to inoculum

Infectivity was greatest when tissue was extracted in 0.3 molar phosphate buffer (Table 4), when sodium phosphate buffers were used in preference to the potassium salts (Table 5), and when buffer pH was about 7.5 (Table 6). Infectivity was increased to varying extents by extracting in the presence of different reducing agents, but the addition of sodium diethyldithiocarbonate to the homogenising buffer, which inhibits the action of oxidising enzymes, reduced infectivity (Table 7). All these results were consistently repeatable.

(c) changes in susceptibility of host

Lesions never developed on C. amaranticolor plants after they commenced flowering. The factor(s) responsible for this apparent immunity are translocated, as leaves on vegetative plants inarch grafted to flowering plants were less susceptible to infection than leaves on vegetative plants grafted to vegetative plants (Table 8). However, aliquots of a TAV inoculum incubated with sap expressed either from flowering or vegetative C. amaranticolor

TABLE 4.

Lesion numbers induced on C. amaranticolor with TAV inocula extracted from tobacco plants with sodium phosphate buffers (pH 7.5) of varying molarity.

Buffer Molarity	Lesion No. / Half Leaf (X)*	Adjusted Log (X+3)
0.01	3.5	0.663
0.04	6.0	0.719
0.10	7.4	0.731
0.20	11.9	0.736
0.30	14.8	0.804
0.40	9.6	0.761
		LSD (5%) 0.059
		LSD (1%) 0.078
		LSD (0.1%) 0.101

* each figure represents the mean number of lesions on 30 half leaves.

TABLE 5.

Lesion numbers induced on C. amaranticolor with TAV inocula extracted from tobacco plants with either sodium or potassium phosphate buffers (0.3 M, pH 7.5).

Buffer Type	Lesion No. / Half Leaf (X)*	Adjusted Log (X+30)
potassium	73.1	1.959
sodium	120.7	2.113
		LSD (0.1%) 0.129

* each figure represents the mean number of lesions on 32 half leaves.

TABLE 6.

Lesion numbers induced on C. amaranticolor with TAV inocula extracted from tobacco plants with 0.3 M sodium phosphate buffers of varying pH.

Buffer pH	Lesion No. / Half Leaf (X)*	Adjusted Log (X+5)
6.0	38.5	1.639
7.0	64.2	1.840
8.0	62.9	1.832
		LSD (5%) 0.126

* each figure represents the mean number of lesions on 30 half leaves.

TABLE 7.

Lesion numbers induced on C. amaranticolor with TAV inocula extracted from tobacco plants with 0.3M sodium phosphate buffer (pH 7.5) containing various inhibitors of polyphenoloxidase activity.

Substance(s) Added*	Lesion No. / Half Leaf*** (X)	Adjusted Log (X+1)
0.01 M DIECA (A)	0.8	0.191
0.01 M cysteine (B)	119.4	1.939
0.01 M ST (C)	1.4	0.272
A + B	98.6	1.819
A + C	1.6	0.356
B + C	118.1	1.899
A + B + C	70.9	1.721
nil	3.4	0.514
	LSD (5%)	0.617
	LSD (1%)	0.823
	LSD (0.1%)	1.077

* A = sodium diethyldithiocarbonate, B = cysteine hydrochloride, and C = sodium thioglycollate

*** each figure represents the mean number of lesions on 14 half leaves.

TABLE 8.

Effects of various treatments to C. amaranticolor on the response to a later inoculation with TAV.

Pre-inoculation Treatment	Mean Lesion No./ Half Leaf (X)	Control for X	Significance
graft to flowering plants	7.4	17.2	< 0.05
dark / 24 hours	108.4	29.4	< 0.001
$36^{o}C$ / 24 hours	4.2	9.4	< 0.05

plants had similar infectivities.

Treatment of C. amaranticolor at 36°C for 24 hours prior to inoculation decreased their susceptibility to infection with TAV. However, a 24-hour pre-inoculation dark treatment greatly increased their susceptibility, and this was repeatable so consistently that it was adopted as a standard procedure (Table 8).

(d) dilution curves

Siegel (1966) briefly reviewed data published on attempts to establish the relationship between virus concentration and lesion number. He concluded that a single virus particle initiates each lesion and that all infectible sites are equally susceptible. Furumoto & Mickey (1967a, 1967b) confirmed this conclusion by applying data they obtained to sophisticated mathematical models. Therefore, the relationship between lesion number and virus concentration seems to be that of a Poisson distribution which may be expressed as:

$$Y = N (1 - e^{-ax})$$

where: Y = number of lesions

N = number of infectible sites

a = constant dependent on the susceptibility of infectible sites

and x = virus concentration

Several experiments were done to test whether the lesion numbers induced on C. amaranticolor by serial dilutions of crude extracts of TAV from tobacco fitted this

relationship. In every instance, by a suitable choice of "N", a plot of "$\ln(1 - \frac{Y}{N})$" against "x" yielded a straight line which passed through the origin. Values of "N" and "a" obtained in these experiments are shown in Table 9, and one of the dilution curves (from experiment no. 4) is shown in Figure 1. This relationship between "Y" and "x" is nonlinear and the form of the curve, determined by the values of "N" and "a", may vary greatly between experiments (Table 9; Kleczkowski, 1950). Therefore the relative infectivities of two or more inocula cannot be assessed accurately unless each is diluted so that they induce equal lesion numbers on opposite leaf halves, or alternatively, serial dilutions of the most infective inoculum are included as treatments in the assay so that "N" and "a" can be estimated.

(e) <u>altering susceptibility of leaves</u>

A preliminary experiment, designed to study the thermal inactivation of TAV, involved assaying the infectivities of 8 inocula on each of 30 half leaves. The first inoculum was applied to all its appropriate half leaves immediately after removing the plants from the dark. This was followed by applying the second inoculum to all its half leaves, etc.. The time taken to complete all the inoculations was 90 minutes. The results of the lesion counts were completely farcical. To test whether this was due to the findings of Helms & McIntyre (1967), who reported that the susceptibility of bean leaves to tobacco

TABLE 9.

Estimates of the parameters "N" and "a" for the equation $Y = N (1 - e^{-ax})$ from the results of five serial dilution experiments.

Experiment No.	Value of "N"	Value of "a"
1	80	5.0
2	130	8.4
3	55	0.8
4	100	2.2
5	120	8.2

FIGURE 1.

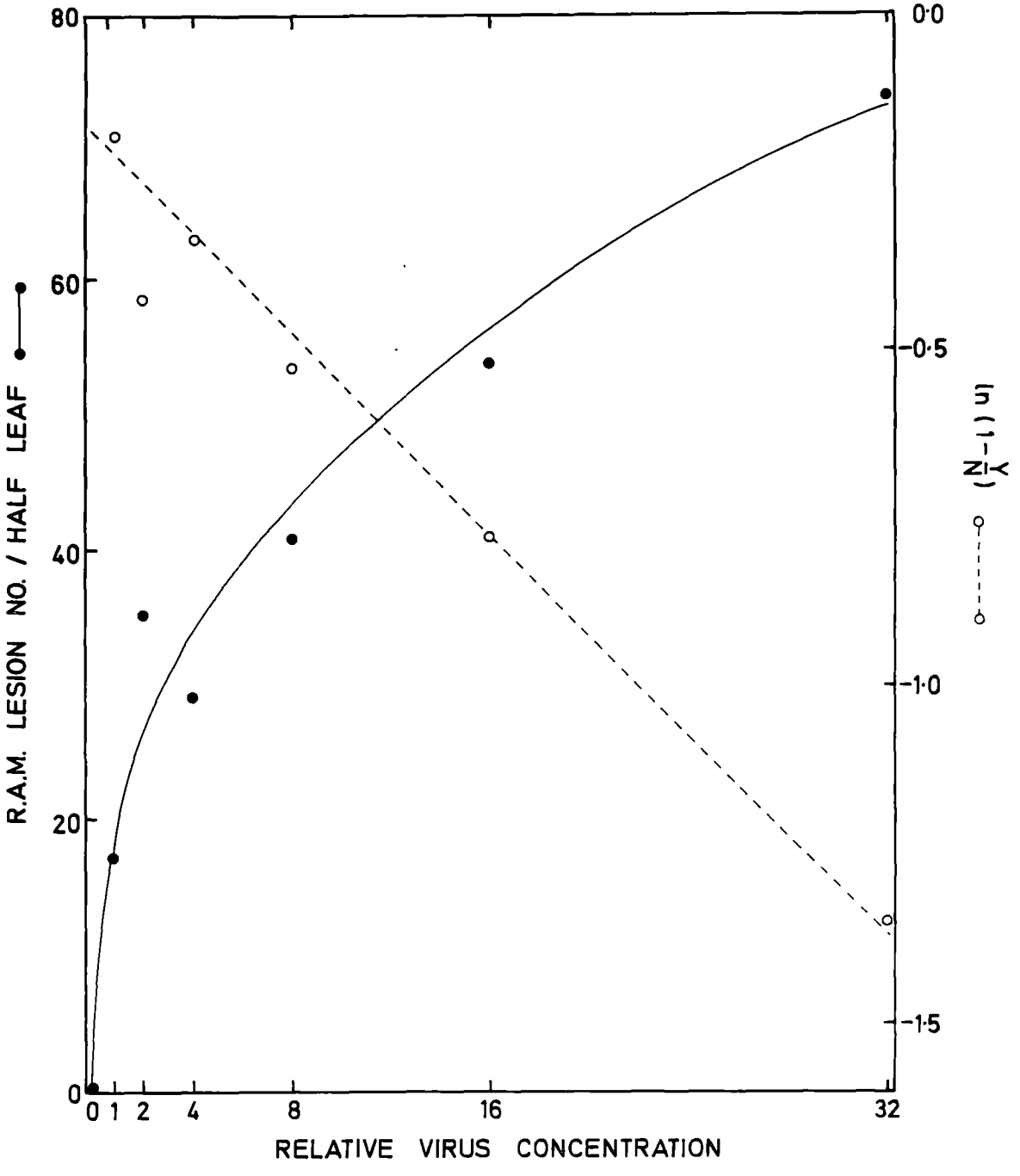
Retransformed adjusted mean* (R.A.M.) lesion numbers induced on C. amaranticolor or by serial dilutions of a TAV inoculum together with a plot of "ln $(1 - \frac{Y}{N})$" against "x"

where: Y = R.A.M. lesion number / half leaf,

N = number of infectible sites / half leaf,

and x = relative virus concentration.

* each point represents the retransformed adjusted mean number of lesions on 30 half leaves.


80
60
40
20
0
0·0
-0·5
-1·0
-1·5
0 1 2 4 8 16 32
R.A.M. LESION NO. / HALF LEAF
RELATIVE VIRUS CONCENTRATION
$(\frac{N}{\lambda}-1)$ u

mosaic virus increased with time of exposure to light following a dark period, six aliquots of the same TAV inoculum, held on ice, were applied to half leaves of 30 C. amaranticolor plants as if they were separate treatments. The 1st aliquot was applied immediately after the plants were removed from the dark to the inoculating room, where the light intensity was about 600 f.c. at the leaf surface. Successive aliquots were applied at 15-minute intervals. The results appeared to verify those of Helms & McIntyre, because the later inoculations induced more lesions than those applied earlier (Figure 2). Another experiment indicated that this effect continued to increase in intensity for up to at least 5 hours. However, to check that these results were not due to effects other than light, the following three treatments were compared:

1) plants held in dark for 24 hours, moved to inoculating room and a half of all leaves inoculated, maintained in inoculating room for a further 90 minutes when the opposite half leaves were inoculated, and then returned to the glasshouse.

2) as for treatment 1 except that a photographic darkroom illuminated with a very low intensity orange light was used in place of the normal inoculating room.

3) plants held in the glasshouse before, between and following the two inoculations.

The later inoculations induced more lesions in every instance (Table 10). Additional tests revealed no

FIGURE 2.

Retransformed adjusted mean* (R.A.M.) lesion numbers induced on half leaves of C. amaranticolor by aliquots of a TAV inoculum applied at varying times after removal of the plants from the dark to the inoculating room.

* each figure represents the retransformed adjusted mean number of lesions on 30 half leaves.

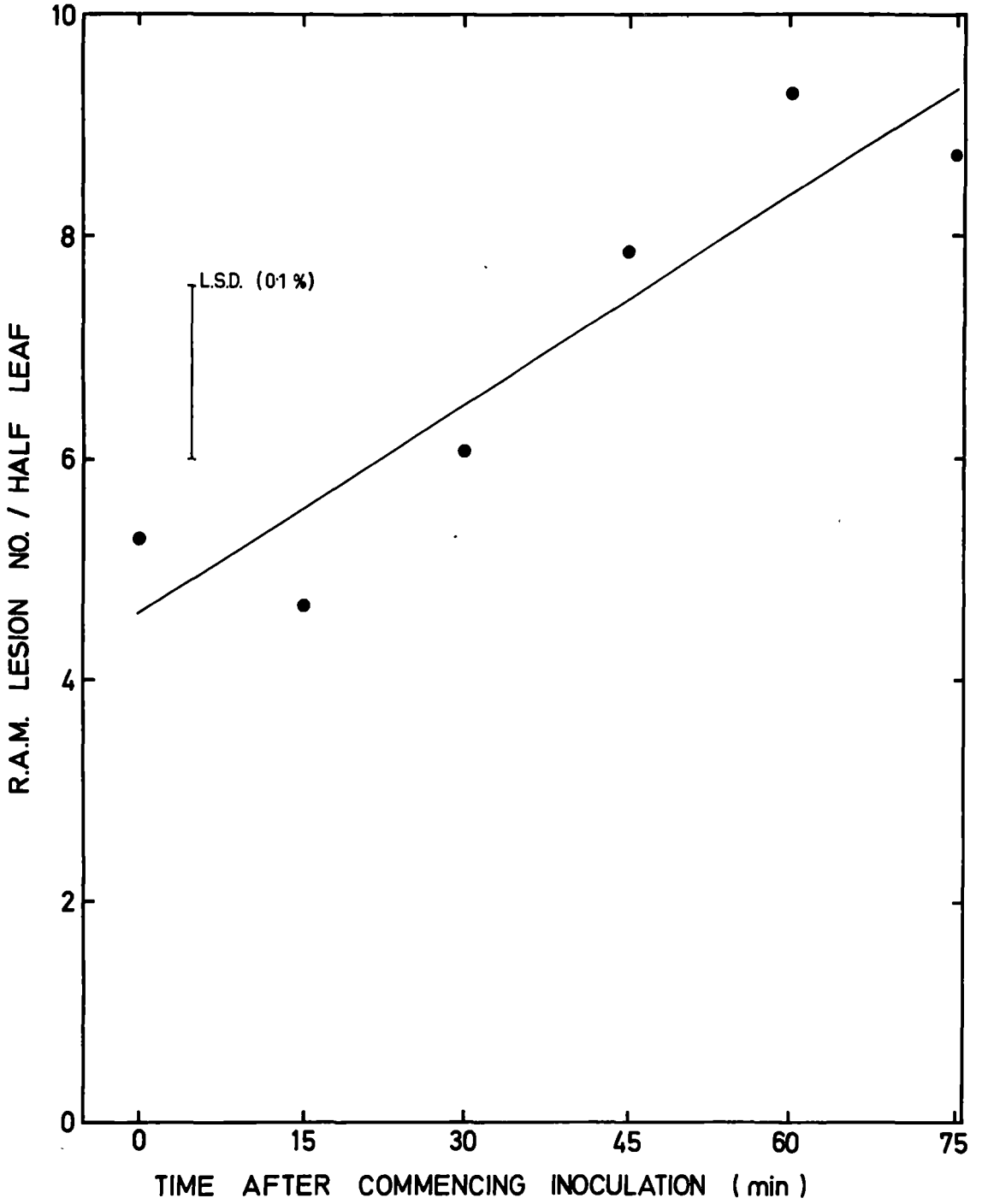

10
8
6
4
2
0
L.S.D. (0·1 %)
R.A.M. LESION NO. / HALF LEAF
0
15
30
45
60
75
TIME AFTER COMMENCING INOCULATION (min)

TABLE 10.

The effect on lesion number of various environmental conditions imposed during a 90 minute interval between inoculating opposite leaf halves of Chenopodium amaranticolor with TAV.

Treatment No.*	R.A.M. Lesion No. / Half Leaf**	
	zero time inoculation	90 minute inoculation
1	4.8	7.0
2	10.3	12.5
3	4.0	8.2

* see text for details (section IIIe).

** R.A.M. = retransformed adjusted mean; differences between inoculations within each treatment were significant at 0.1%; valid comparisons can not be made between treatments.

differences in lesion number when all leaf halves on a plant were inoculated either at zero time or at the 90-minute time under the conditions prevailing in treatments 1 and 3. Furthermore, under treatment 1 conditions, the same effect on the 90-minute TAV inoculation was noted irrespective of whether the zero time treatment on the opposite leaf half had been a TAV inoculation, a potato virus X inoculation, or an inoculation with buffer alone (Table 11). Therefore, it was merely the abrasion of one half of a C. amaranticolor leaf which increased the susceptibility of the opposite half to infection with TAV at a later stage.

The increased susceptibility of uninoculated half-leaves was positively correlated with the concentration of peroxidases within them (Table 12). These estimations were done by triturating the leaf tissue (1:200, W:V) in 0.15 M phosphate buffer (pH 6). One ml of the homogenate, after filtering, was mixed with 3 ml 20 mM pyrogallol and 0.5 ml 1% H_2O_2. The time taken for the transmission of light of wavelength 430 nm to decrease from 80% to 50% was taken as a measure of peroxidase activity. The method was a modification of that described by Chance & Maehly (1955).

Polyphenoloxidase activity was never detected in this species when D-catechin was used as a substrate.

(f) assay procedure adopted

C. amaranticolor plants were raised in a

TABLE 11.

The effect on lesion number of various types of inoculations on one leaf half of Chenopodium amaranticolor on the response of the opposite half to inoculation with TAV 90 minutes later.

Zero Time Treatment	R.A.M. Lesion No. / Half Leaf*
TAV inoculation	63.8
PVX inoculation	61.0
water inoculation	61.0

* R.A.M. = retransformed adjusted mean; there were no significant differences between the treatment means.

TABLE 12.

Relative peroxidase levels in Chenopodium amaranticolor leaves treated in various ways.

Leaf Type	Time (sec.)*
uninoculated leaves at zero time	79.2
inoculated leaf half at 90 minutes	49.0
uninoculated leaf half at 90 minutes	42.4

* The time taken for the transmission of a mixture of 1 ml leaf homogenate, 3 ml 20 mM pyrogallol and 0.5 ml 1% H_2O_2 to decrease from 80% to 50% when measured at 430 nm (peroxidase concentration is inversely correlated with time).

glasshouse and illuminated for 1 hour during the night to prevent flowering (Thomas, 1961). One day before assay, the plant apices and leaves not to be used in the test were removed. The plants were then dusted with 500 mesh carborundum and placed in the dark. Unless stated otherwise, samples for measurement of infectivity were prepared by triturating leaf tissue at 0°C (1:1, W:V) in cold 0.3 M sodium phosphate buffer (pH 7.5) containing 0.01 M cysteine hydrochloride. The homogenates were squeezed through cheesecloth and applied with the index finger to half leaves of C. amaranticolor so that, on any one plant, there was never more than a 10-minute interval between the first and last inoculations. Each half leaf was rinsed with tap water immediately after the inoculum was applied to it.

Comparisons of more than two samples were done by using balanced incomplete block experiments (Cochran & Cox, 1957). Individual plants were used as replicates, and leaves as blocks. The designs always allowed differences between leaf age response to be calculated. At least 12 replicates were included in every assay. Whenever possible, experiments were designed so that information was not required on the precise relative infectivities of the inocula, but only on whether they were more, or less, infective than each other. If precise relative infectivities were required, dilutions of the most infective inoculum were included as treatments

in the experimental design. However, this seriously limited the number of comparisons which could be made as plants with more than 3 to 4 leaves suitable for inoculation were rarely available. Statistical analyses of the local lesion counts were done on a manual Facit calculator (Cochran & Cox, 1957; Fisher & Yates, 1963) after transformation according to the method of Kleczkowski (1949).

IV INHIBITORS AND VIRUS MEASUREMENT

Inhibitors of virus infection, whose production and concentration may be influenced by many different factors, frequently occur in plant extracts (Bawden, 1954). No studies have been made on the effect of the temperatures used for heat treatment (35-40^{o}C) on the production and stability of this group of inhibitors. However, the possibility existed that any measured, apparent effect of short term heat treatments could be ascribed erroneously to effects on TAV, when the real situation was a heat induced change in the level of inhibitors which interferred with measurement of virus concentration. Some experiments were done, therefore, to establish whether inhibitors that interfere with virus assay are present in TAV infected tobacco plants and if there were, whether their concentration changed during heat treatment.

(a) infectivity changes with purification

There was no indication that TAV infected tobacco leaves contained an inhibitor of the type present in

Phytolacca esculenta (Bawden, 1954) whose effect is diminished by dilution (Figure 1).

In two instances, TAV was purified from tobacco as described in Section II(d) and the infectivities of four inocula, representing different stages of purification, were compared. The results showed that apparent infectivity increases during the purification procedure (Table 13), indicating that inhibitors of infection are present in tobacco leaf extracts which are progressively removed during the purification procedure.

(b) effect of chloroform concentration

It seemed possible that the increase in infectivity following chloroform emulsification was due to the release of virus from within chloroplasts and from attachment to membranes which was not extracted by the normal trituration procedure. However, this could not account for its full effect as the infectivity of leaf homogenates was increased merely by low speed centrifugation (Table 14).

Chloroform destroyed some infectivity if too large a proportion was used, but chloroform beyond 0.45 volumes was without further effect (Table 15). Therefore, all further chloroform clarifications were done with 0.10 volumes which was the minimum proportion to give a satisfactory emulsion.

(c) competitive effects of inhibitor

The data in Tables 13, 14 and 15 suggest that

TABLE 13.

The infectivities of TAV samples after various stages of purification.

Sample	T.A.M. Lesion No. / Half Leaf*	
	1st experiment	2nd experiment
leaf homogenate	98.1	1.918
chloroform clarified	197.3	2.145
P2**	250.3	2.662
P3**	189.6	2.578
LSD (5%)	41.6	0.192
LSD (1%)	57.4	0.280
LSD (0.1%)	78.9	-

* T.A.M. = transformed adjusted mean; no transformation was necessary in the first experiment; the transformation was log (X+20) in the second experiment; each figure was derived from the number of lesions on 12 half leaves.

** P2 and P3 refer to the virus resuspended in buffer after the second and third high speed centrifugations, respectively. These inocula were diluted with buffer so that they were directly comparable with the other two treatments.

TABLE 14.

The infectivities of TAV in aliquots of a tobacco leaf homogenate filtered through cheesecloth before and after clarification by low speed centrifugation (10,000 g/10 min).

Treatment	R.A.M. Lesion No. / Half Leaf*	
	1st experiment	2nd experiment
centrifuged	113.5	3.36
not centrifuged	78.8	2.24
level of significance	0.1%	1%

* R.A.M. = retransformed adjusted mean; each figure was derived from the number of lesions on 24 half leaves.

TABLE 15.

Infectivities of aliquots of TAV leaf homogenates clarified with varying proportions of chloroform.

Proportion Chloroform Added	T.A.M. Lesion No. / Half Leaf*	
	1st experiment	2nd experiment
0.15	2.143	1.067
0.30	2.085	0.778
0.45	1.891	0.540
0.60	1.943	0.769
LSD (5%)	0.081	0.144
LSD (1%)	0.111	0.197
LSD (0.1%)	0.153	0.267

* T.A.M. = transformed adjusted mean; the transformations were log (X+50) and log (X+1) in the first and second experiments, respectively; each figure was derived from the number of lesions on 12 half leaves.

successive stages of purification remove substances which reduce infectivity by competing with the virus for infectible sites on the assay host. I attempted to obtain rigorous proof of this point by using the principles of Michaelis-Menten enzyme kinetics by assuming that:

(i) chloroform clarified infected sap (CS) is equivalent to substrate (S)

(ii) healthy unclarified sap (HS) is equivalent to inhibitor

(iii) infectible sites are equivalent to the enzyme molecules

(iv) lesion number is equivalent to the initial reaction velocity (V)

The infectivities of six inocula containing various proportions of CS, HS and buffer (see Table 16 for details of the composition of these inocula) were compared so that two plots could be made of I/V against I/S to determine whether or not competitive inhibition occurs.

The local lesion counts indicated that inocula diluted with healthy sap were more infective than those diluted with buffer (Table 16). Therefore, these results failed to confirm the hypothesis and, in contradiction, suggested that the buffer competed with TAV for infectible sites. This can be seen in Figure 3 where a Michaelis-Menten plot of I/V against 1/(1-S) is set out for inocula numbers 2, 3, 4 and 5. Later tests showed that the healthy sap extracts used in this experiment did not induce local lesions by themselves.

TABLE 16.

The infectivities of inocula containing varying proportions of buffer, healthy plant sap (HS) and chloroform clarified infected sap (CS).

Inoculum No.	Relative Proportions of			T.A.M. Lesion No. / Half Leaf*
	CS	HS	buffer	
1	1.00	-	-	2.040
2	0.50	-	0.50	1.846
3	0.33	-	0.67	1.573
4	0.50	0.50	-	1.881
5	0.33	0.50	0.17	1.766
6	0.25	0.50	0.25	1.745
			LSD (5%)	0.181
			LSD (1%)	0.243
			LSD (0.1%)	0.320

* T.A.M. = transformed adjusted mean; the transformation was log (X); each figure in the last column was derived from the number of lesions on 15 half leaves.

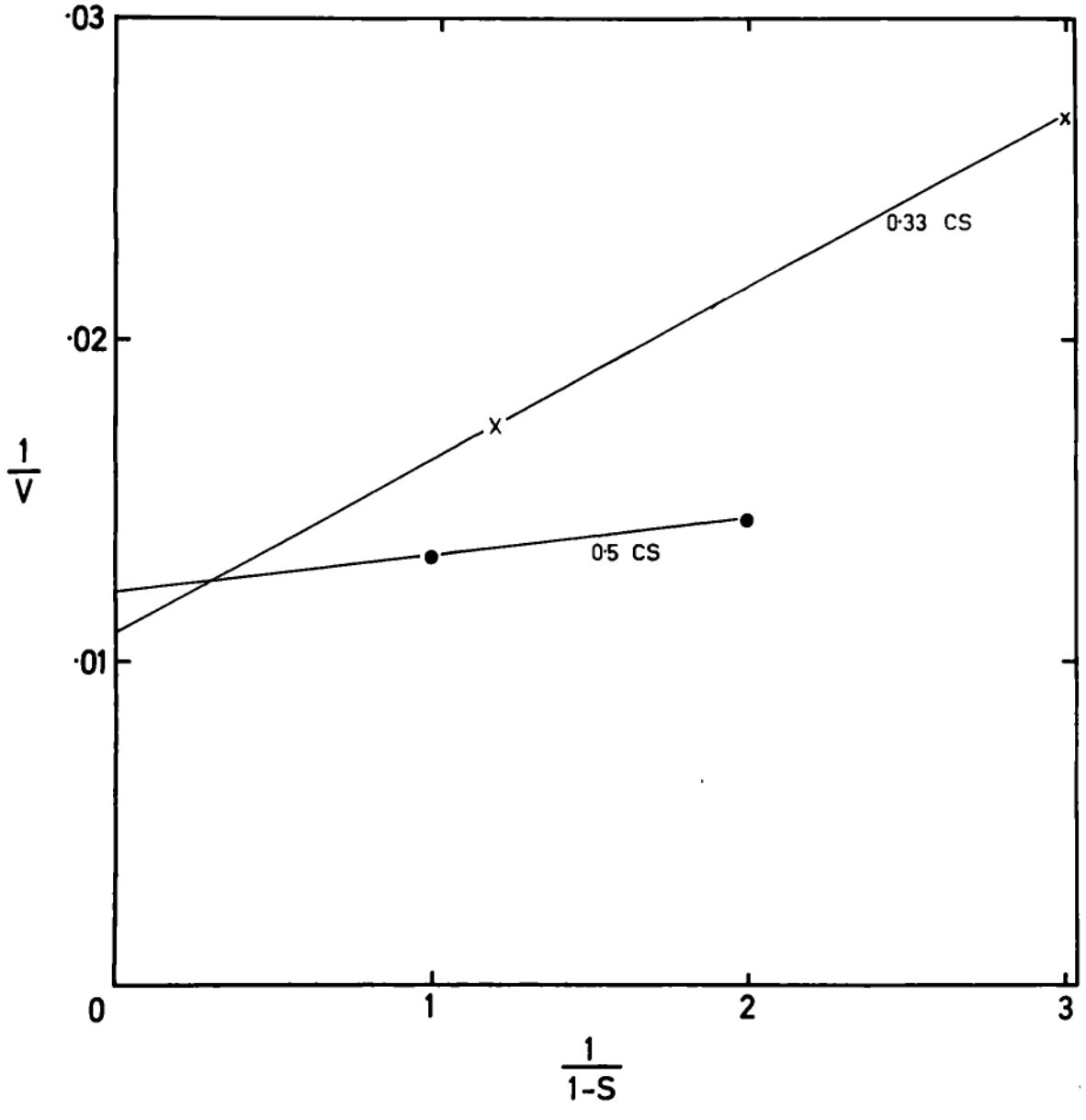
FIGURE 3.

The relationship between I/V against $\frac{I}{I-S}$ for two concentrations of CS,

where: V = the initial reaction velocity (equivalent to retransformed adjusted mean lesion number / half leaf),

S = the proportion of substrate in the inoculum (equivalent to proportion of chloroform clarified infected sap),

and CS = proportion of chloroform clarified infected sap.


·03
·02
·01
0
1
2
3
1/V
1/1-S
0·33 CS
0·5 CS
x
x

(d) factors affecting inhibitor concentration

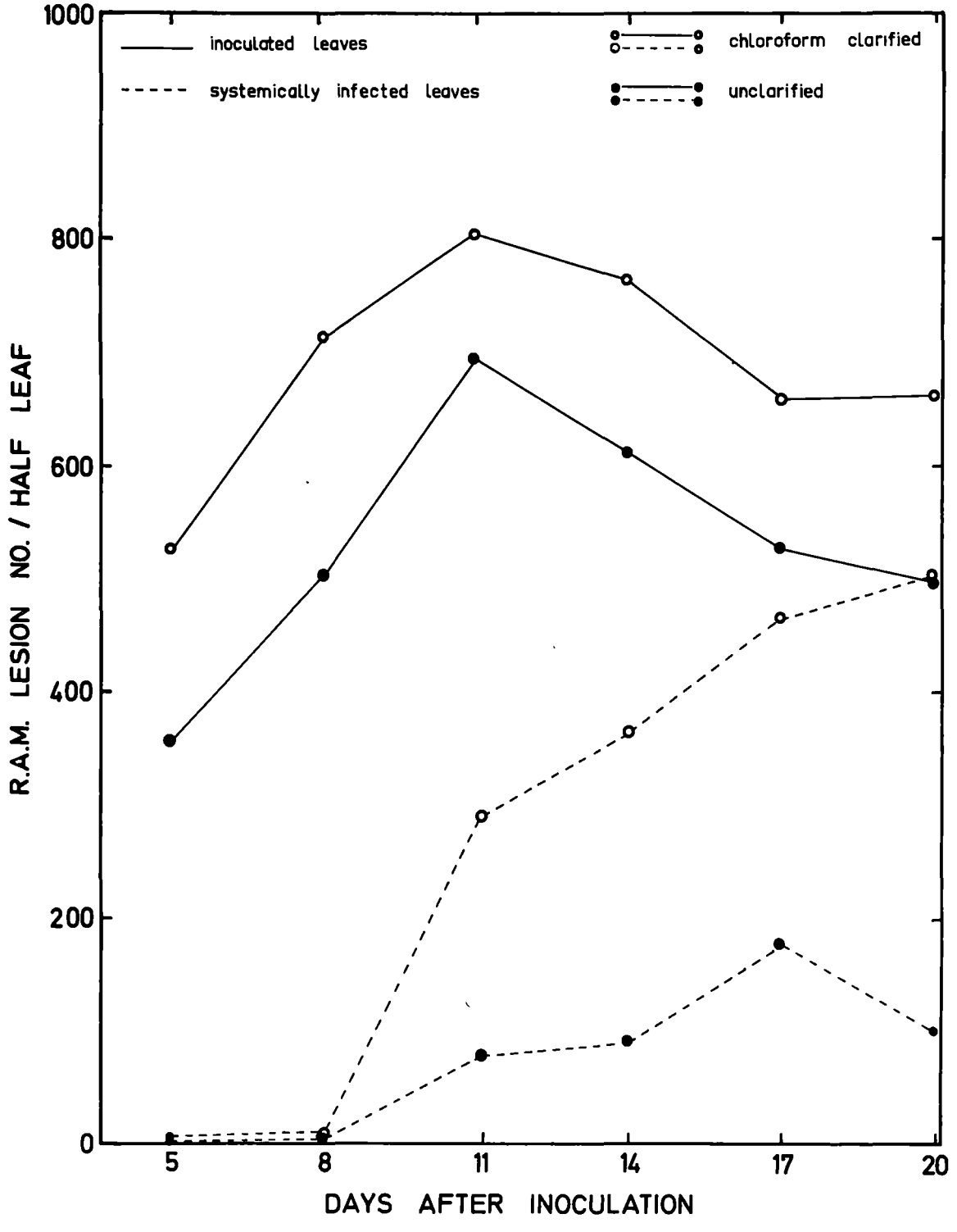
Results from the preceding section indicate that inhibitor removed by chloroform probably was present only in virus infected plants. Also, the extent to which chloroform clarification increased apparent infectivity varied greatly between experiments which suggested that the concentration of inhibitor may vary according to leaf age and the time lapse since inoculation.

These points were checked by inoculating the most fully expanded leaf on a batch of young tobacco plants with TAV. Five days later, the inoculated leaves from 20 plants were triturated, lyophilised and stored under vacuum at $-10^{o}C$. All leaves above that which was inoculated (systemically infected leaves) on these plants were treated, separately, in the same way. This procedure was repeated at 3-day intervals up to 20 days after inoculation. Later, the lyophilised homogenates were reconstituted with water and portions of them clarified with 0.10 volumes of chloroform. All samples were then assayed for infectivity and serological titre.

The results showed that the level of infective virus reached far higher levels in inoculated than in systemically infected leaves, and that the increase in apparent infectivity following clarification is much greater for systemically infected than for inoculated leaves (Figure 4). In both leaf types, the efficiency of chloroform clarification in removing inhibitor increased

FIGURE 4.

The retransformed adjusted mean (R.A.M.) lesion numbers / half leaf induced on C. amaranticolor by homogenates derived from inoculated and systemically infected tobacco leaves at various times after inoculation. Aliquots of each homogenate were compared both before and after clarification with chloroform.


1000
800
600
400
200
0
R.A.M. LESION NO. / HALF LEAF
inoculated leaves
systemically infected leaves
chloroform clarified
unclarified
5
8
11
14
17
20
DAYS AFTER INOCULATION

with time after inoculation. A peak of infectivity occurred in both leaf types while serological titre maintained a plateau after the peak was reached. Another observation was that visual symptoms of virus infection in both types of leaf were very mild at the time when the levels of infective virus within them had reached their peaks. Symptoms became much more intense some time later, when the concentration of infective virus had fallen.

These findings necessitated that, as far as possible, quantitative measurement of infectivity changes in whole plants during heat treatment should be done over relatively short time periods so that significant changes in the proportion of types of leaf would not occur, and also, so that natural declines in infectivity following the peak would not be attributed to a heat treatment effect.

(e) thermolability of inhibitor

Infected plants were heat treated for periods of up to five hours. The leaves were then triturated in the normal way and the infectivities of the homogenates compared before and after clarification with chloroform. As well, infected leaf homogenates were heat-treated in stoppered test tubes for varying periods and their infectivities compared with those of subsamples clarified after heat treatment. In these experiments, each inoculum was derived from a bulked sample of the leaves from 16 plants.

It was presumed that a change in the concentration of inhibitor during heat treatment would result in an

alteration of the ratio between the infectivities of clarified and unclarified inocula, and was checked by the chi-square test (Snedecor, 1956). The results indicated no change in inhibitor level during heat treatment (Table 17).

(f) inhibitors affecting serological assay

The serological titre was determined for each of the samples tested for infectivity, as outlined in the previous section. These results are set out in Table 18. They show that inhibitor was present which either combined with TAV and retarded movement out of the well, or combined with some antigenic sites on TAV so that the antiserum could not combine with some of the virus particles. The inhibitor was very heat labile and was also destroyed by clarifying with chloroform.

The following virus preparations were then tested against 20 arithmetical dilutions of antiserum from 1/10 up to 1/500 to determine whether this distinct heat labile inhibitor which interfered with serological assays was either virus-induced or a normal constituent of tobacco plants.

Preparation No.	Virus Preparation
1	unheated clarified infected sap (CS)
2	CS diluted 1:1 with buffer
3	CS diluted 1:1 with healthy sap heated at $36^{o}C$/5 hours
4	CS diluted 1:1 with unheated healthy sap
5	unheated unclarified infected sap (US)
6	US diluted 1:1 with buffer

TABLE 17.

Comparisons between the amount of inhibitor removed by chloroform from leaf homogenates after different periods of heat treatment 'in vivo' and 'in vitro'.

Experiment No.	Medium	Treatment Times (hours)	Chi Square
1	expressed sap	0, 1, 2, 3	0.76 (N.S.)*
2	expressed sap	3, 4, 5	3.91 (N.S.)
3	intact plant	0, 1, 2	0.38 (N.S.)
4	intact plant	3, 4	0.00 (N.S.)

* denotes 'not significant'.

TABLE 18.

Virus titres of leaf homogenates before and after chloroform clarification following various periods of heat treatment 'in vivo' and 'in vitro'.

Treatment Time (hours)	Virus Titre			
	Medium for Heat Treatment			
	intact plant		expressed sap	
	unclarified	clarified	unclarified	clarified
0	32	128	32	128
1	64	128	64	128
2	64	128	64	128
3	128	128	64	128
4	128	128	64	128
5	no test done		128	128

Visible precipitin lines were produced against all preparations at all antiserum dilutions. However, estimates of the apparent relative antigenicity of the six preparations were gauged by observing the relative distance of the lines between antigen and antiserum wells. These observations indicated that the apparent virus concentrations of preparations 1 and 2 were greater than those of 5 and 6, respectively. There was no difference between preparations 3 and 4, but the apparent virus concentration of these was similar to that of preparation 2 and greater than that of preparation 6. Therefore, the inhibitor was present only in infected plant tissue, and the previous results on the thermolability of this inhibitor were confirmed.

V THERAPY OF TAV INFECTED PLANTS BY HEAT TREATMENT

Four separate experiments were done to investigate the length of heat treatment required to cure TAV infected tobacco plants. In each experiment, infected plants were placed in the phytotron cabinet 14 days after inoculation when all showed obvious symptoms of infection. Batches of ten plants were returned to the glasshouse at 5-day intervals. The longest period of treatment was 30 days. Immediately after removal from the cabinet, the above-ground portion of each plant was cut off, rooted in a mist propagation unit, and then grown on in the glasshouse (except that no cuttings were taken from plants treated in the first experiment or from the plants treated for 5-20 days in the second experiment). The original plants and the

rooted cuttings were inspected at regular intervals for symptoms of infection. Those plants which had not developed symptoms within 9 months following treatment were inoculated with TAV to determine whether they were infected with latent attenuated virus strains.

The results from the four experiments are presented in Tables 19-22. In the 1st and 4th experiments, no cures were obtained with less than 15 days treatment and the proportion of cures increased with duration of treatment beyond this time. In the 4th experiment, resurgence of symptoms in plants not completely cured was more rapid than in plants treated for shorter periods of time (this information was not recorded in the 1st experiment). The results from the 2nd and 3rd experiments were completely different. Here, some cures were obtained with very short periods of treatment and the proportion of cures was often greater with shorter than with longer treatments. Also, resurgence of symptoms in plants not completely cured was more rapid in plants treated for longer than for shorter periods of time. A feature common to the last three experiments was that the proportion of cures effected after any period of treatment was greater for rooted cuttings than for the original plants from which the cuttings were taken. However, in one instance (in the 3rd experiment), an original plant was freed from infection by heat treatment although the cutting taken from it proved to be infected.

The results of these experiments, particularly the

TABLE 19.

Therapy of TAV infected tobacco plants by heat treatment (1st experiment).

Duration of Treatment (days)	Proportion Healthy*
5	0/10
10	0/10
15	1/10
20	3/10
25	10/10
30	10/10

* numerator represents the number of plants freed from infection and denominator the total number of plants which survived the treatment.

TABLE 20.

Therapy of TAV infected tobacco plants by heat treatment (2nd experiment).

Treatment Time (days)	Original Plants		Rooted Cuttings	
	cures*	days to symptoms**	cures*	days to symptoms**
5	1/10	73		
10	1/10	66		
15	0/9	61		
20	1/10	66		
25	0/10	53	5/5	-
30	0/10	43	2/9	76

* numerator represents the number of plants freed from infection and denominator the number of plants which survived the treatment.

** mean number of days from conclusion of treatment until virus symptoms were observed on plants not freed from infection.

TABLE 21.

Therapy of TAV infected tobacco plants by heat treatment (3rd experiment).

Treatment Time (days)	Original Plants		Rooted Cuttings	
	cures*	days to symptoms**	cures*	days to symptoms**
5	3/10	108	4/8	110
10	4/10	103	9/10	116
15	8/9	85	8/9	85
20	3/10	56	9/10	106
25	5/10***	40	6/7	184
30	9/10	70	10/10	-

* numerator represents the number of plants freed from infection and denominator the number of plants which survived the treatment.

** mean number of days from conclusion of treatment until virus symptoms were observed on plants not freed from infection.

*** the top cutting from one of these plants was infected.

TABLE 22.

Therapy of TAV infected tobacco plants by heat treatment (4th experiment).

Treatment Time (days)	Original Plants		Rooted Cuttings	
	cures*	days to symptoms**	cures*	days to symptoms**
5	0/10	19	0/10	76
10	0/10	67	0/10	71
15	3/10	75	9/10	141
20	5/10	93	9/9	-
25	5/10	77	5/5	-
30	10/10	-	7/7	-

* numerator represents the number of plants freed from infection and denominator the number of plants which survived the treatment.

** mean number of days from conclusion of treatment until virus symptoms were observed on plants not freed from infection.

2nd and 3rd, provide evidence that therapy by heat treatment cannot be accounted for solely on the basis of a direct thermal effect on TAV because there was no correlation between either the time of treatment and % of cures, or between the time of treatment and the rate at which TAV multiplied in plants not freed from infection after removal from the cabinet.

VI NET CHANGES IN PLANT PROTEIN CONTENT DURING HEAT TREATMENT

The results from the preceding section show that TAV is often eliminated from infected plants either during heat treatment, or following heat treatment, or both. However, they do not indicate whether this is due to an increased rate of inactivation, a decreased rate of synthesis, a combination of both these factors, or a greater increase in the rate of inactivation than the increase in rate of synthesis. I could not devise experiments to solve this query directly at the time, and therefore turned to examine overall plant protein synthesis because the pathways of synthesis and degradation of host proteins closely ally those of viral synthesis and inactivation (Weissmannet al., 1966; Bosch et al., 1966). The first aspect studied was to examine the changes in the total and protein nitrogen contents of heated and control plants.

Infected plants were placed in the phytotron cabinet 14 days after inoculation together with similar uninoculated plants. Control plants were grown on in the glasshouse. Half of all plants were sprayed daily with

10^{-4} M kinetin (Calbiochem). These, and all future sprayings were done with a vacuum cleaner fitted with an aerosol venturi sprayer, to insipient run-off. The spray treatment was included in the experiment because Engelbrecht & Mothes (1960) reported that this compound increased the heat resistance of Nicotiana rustica, and because it stimulates protein synthesis in senescing leaf tissue (e.g. Osborne, 1962). Batches of five plants from each environment-infection type-spray treatment combination were harvested at 5-day intervals up to 20 days. After drying, the weights of individual plants were found to be too small for them to be analysed for total and protein nitrogen content, and average analyses therefore had to be done on combined 5-plant samples. Total nitrogen content was determined by the micro-kjeldahl method of Markham (1942) following digestion of dried leaf tissue with sulphuric acid, sodium sulphate and copper sulphate. Protein nitrogen was estimated after extraction of soluble nitrogen from the tissue (1:100, W:V) with 0.3 M sodium chloride in 0.025 M sodium phosphate at pH 7.0 in an end over end shaker at 1^{o}C for 24 hours (Oland, 1959). Determination of each sample was done in duplicate and checks on analytical technique were made frequently by subjecting sucrose blanks and known quantities of ammonium sulphate to the procedure.

The results, presented in Figures 5-7, were averaged over healthy and infected plants as there was no

FIGURE 5.

The total and protein nitrogen contents (expressed on a % dry weight basis) of tobacco plants after varying periods of heat treatment when sprayed daily with either 10^{-4} M kinetin or with water, compared with that of control plants grown in the glasshouse.

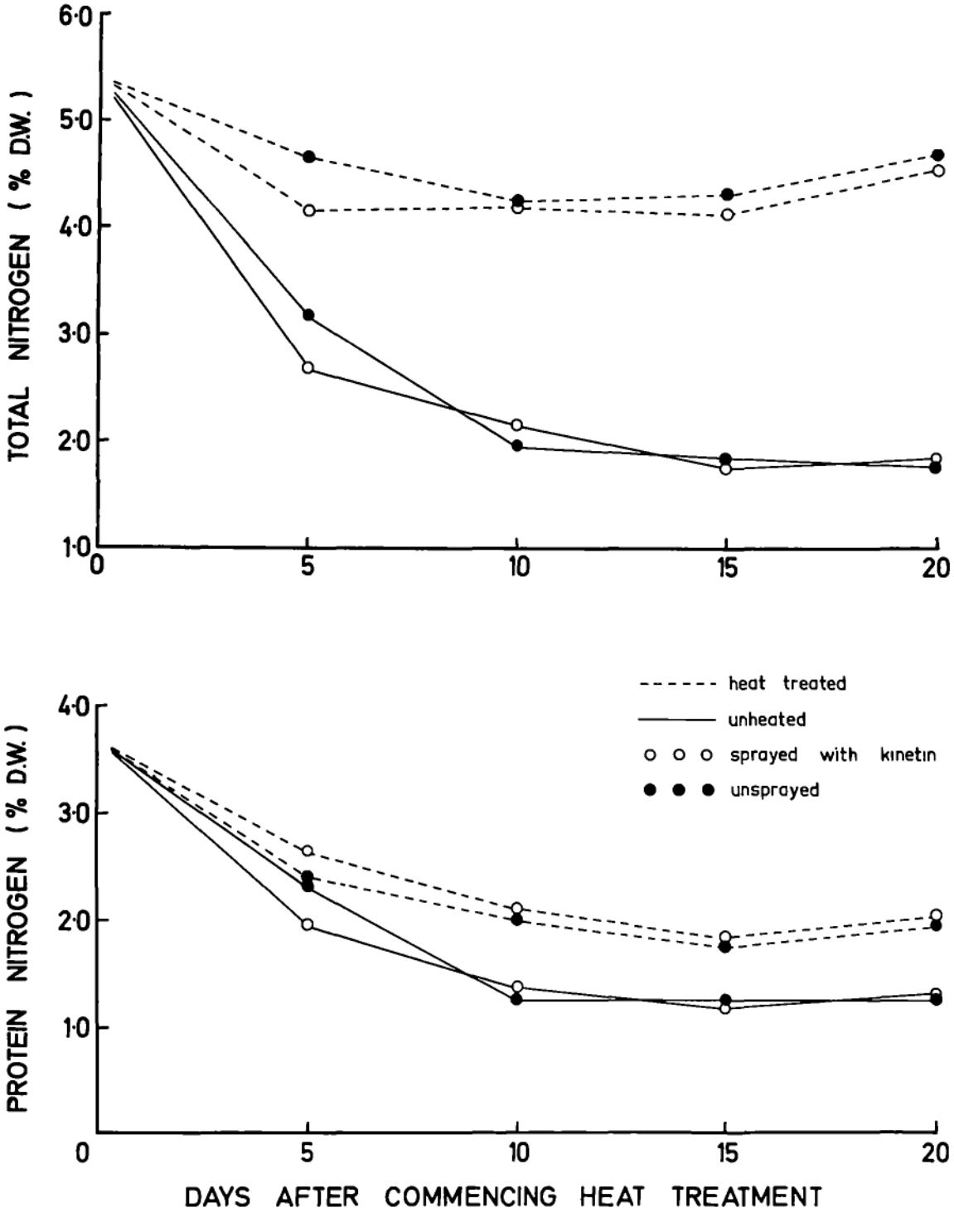

TOTAL NITROGEN (% D.W.)
6·0
5·0
4·0
3·0
2·0
1·0
0
5
10
15
20
heat treated
unheated
sprayed with kinetin
unsprayed
PROTEIN NITROGEN (% D.W.)
4·0
3·0
2·0
1·0
0
5
10
15
20
DAYS AFTER COMMENCING HEAT TREATMENT

FIGURE 6.

The proportion of protein nitrogen to total nitrogen and the dry weight of tobacco plants after varying periods of heat treatment when sprayed daily with either 10^{-4} M kinetin or with water, compared with that of control plants grown in the glasshouse.

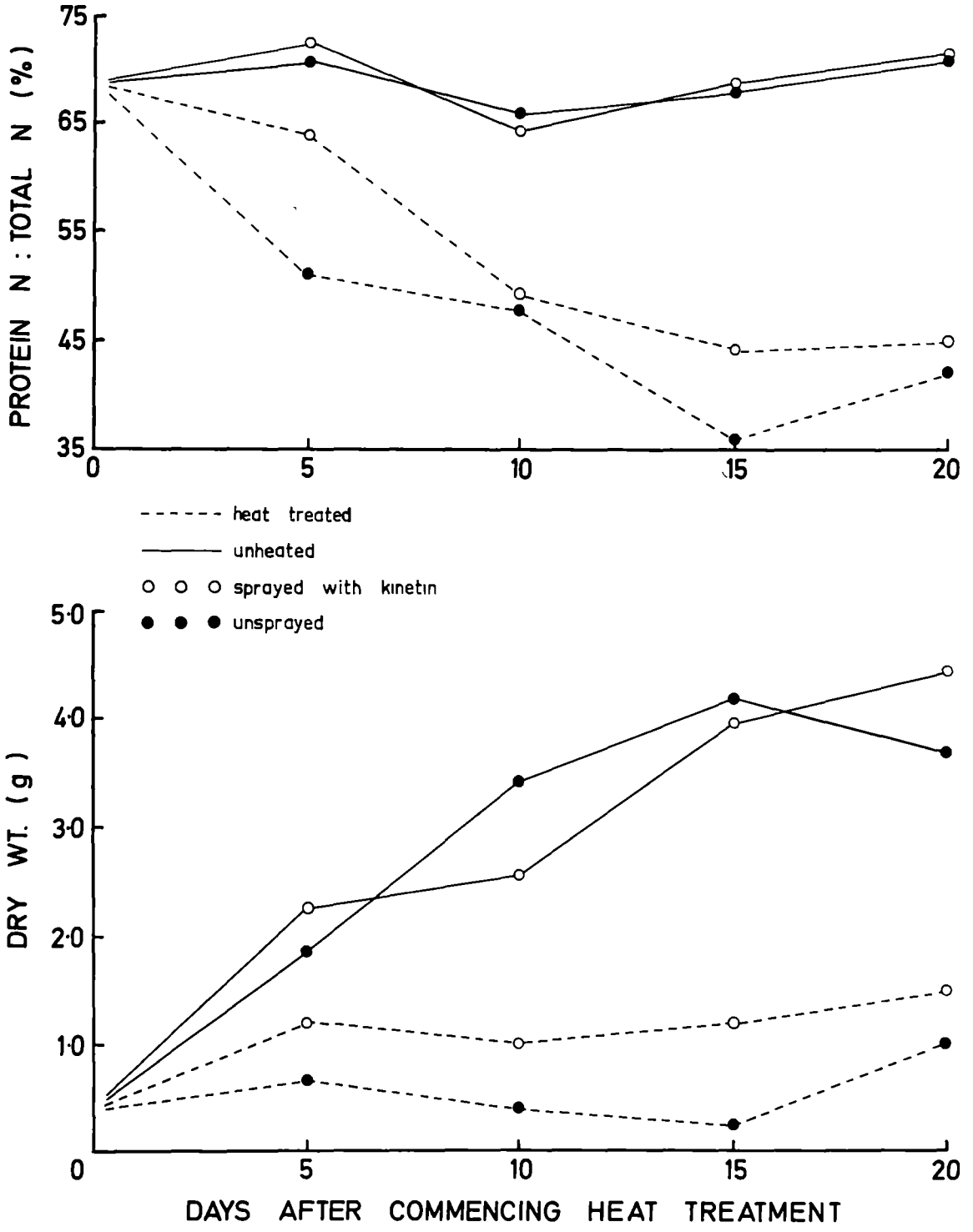
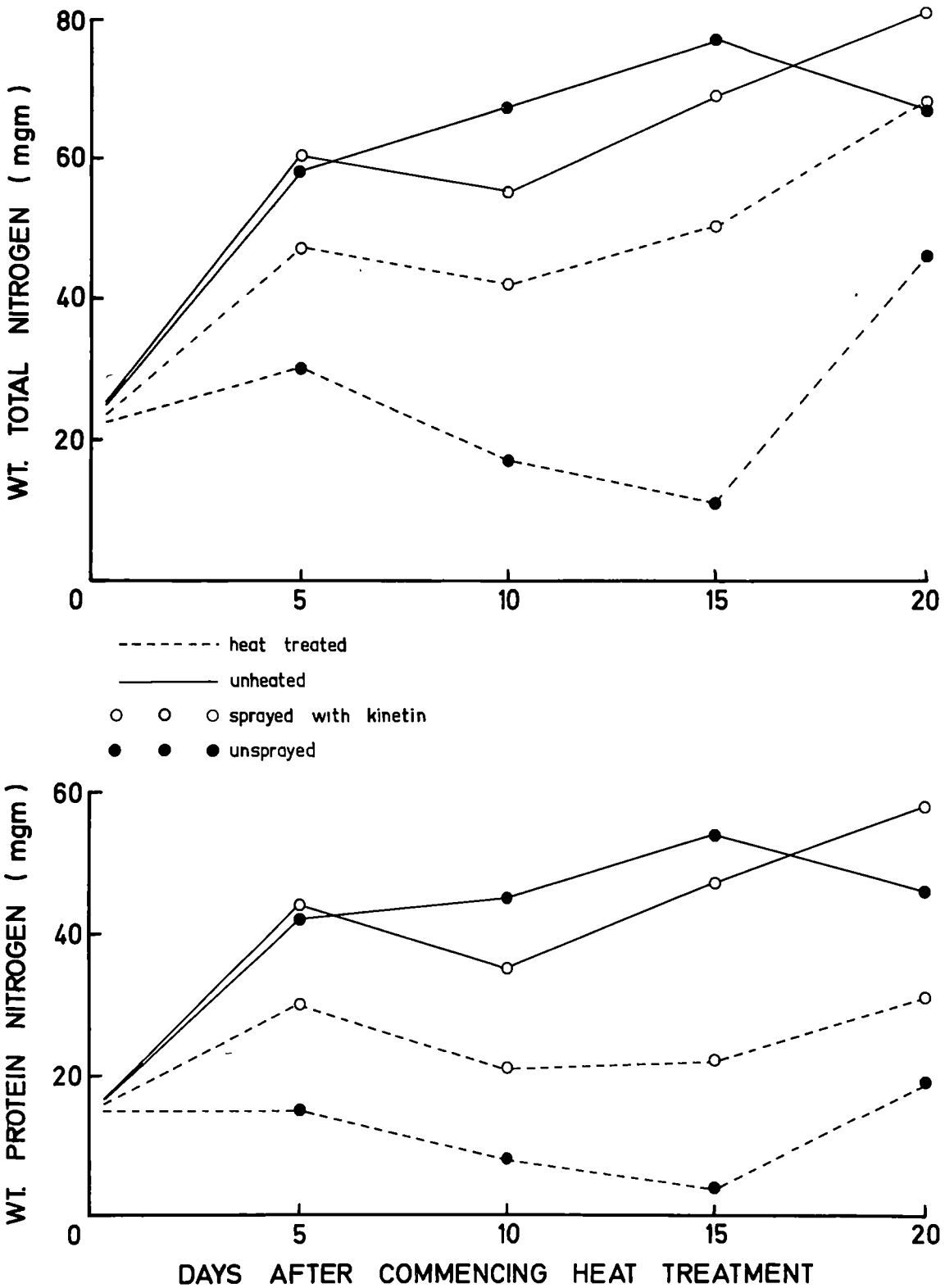

PROTEIN N : TOTAL N (%)
75
65
55
45
35
0
5
10
15
20
heat treated
unheated
sprayed with kinetin
unsprayed
DRY WT. (g)
5·0
4·0
3·0
2·0
1·0
0
5
10
15
20
DAYS AFTER COMMENCING HEAT TREATMENT

FIGURE 7.

The absolute total nitrogen and protein nitrogen contents of tobacco plants after varying periods of heat treatment when sprayed daily with either 10^{-4} M kinetin or with water, compared with that of control plants grown in the glasshouse.


WT. TOTAL NITROGEN (mgm)
80
60
40
20
0
5
10
15
20
heat treated
unheated
sprayed with kinetin
unsprayed
WT. PROTEIN NITROGEN (mgm)
60
40
20
0
5
10
15
20
DAYS AFTER COMMENCING HEAT TREATMENT

obvious interaction between infection level and any other variant. Total and protein nitrogen contents of unheated plants, on a % dry weight basis, decreased with time; this was associated with a change from the rosette form to stem elongation and flower initiation during the period of the experiment. However, absolute total nitrogen content increased about four-fold during the 20-day period of the experiment and a constant proportion of this was maintained in protein. Spraying with kinetin was without effect. In heated plants, the proportion of protein to total nitrogen decreased markedly with time of treatment. The plants made no significant growth in terms of dry weight increase and it appeared that absolute protein content may have decreased with time. Kinetin partially reversed these effects, as heated plants sprayed daily with kinetin increased in dry weight, increased their absolute protein content, and maintained a greater proportion of their nitrogen in the protein form. In a subsidiary investigation, healthy heated plants sprayed daily with 10^{-4} M benzyladenine (Calbiochem), a synthetic cytokinin chemically related to kinetin, were compared with others sprayed either with 10^{-4} M kinetin or with water. The results, shown in Table 23, indicate that the status of plants sprayed with benzyladenine was intermediate between that of the water and kinetin-sprayed plants.

The results of the above experiment, summarised in Figures 5-7, of necessity were done on bulked plant

TABLE 23.

Dry weight and nitrogen status of healthy tobacco plants heated at 36°C/5-20 days when sprayed daily with either water, kinetin or benzyladenine.

Parameter	Spray Treatment		
	water	benzyladenine	kinetin
dry weight (g)	0.68*	1.16	1.38
total N (% D.W.)	4.88	4.32	4.44
protein N (% D.W.)	2.38	2.43	2.51
protein N : total N (%)	47.9	56.3	56.5
wt. total N (mgm)	33.2	50.1	61.3
wt. protein N (mgm)	16.2	28.2	34.6

* each figure represents the mean of 2 samples taken at intervals of 5 days after commencing heat treatment.

samples which meant that there was no effective replication and that the results could not be statistically analysed. Therefore, each individual result may have been weighted unduly by any extremes within the 5-plant samples, although this was not obviously apparent through any erratic trends in the graphs apart, perhaps, from those concerned with the dry weights of heat treated plants. The experiment was therefore repeated, identical in design, except that a complete benzyladenine spray treatment was included, and that the treatments were first applied when the plants were larger so that each could be analysed individually.

The results of this experiment are summarised in Figures 8 and 9 while the full details are set out in Appendix 3. Sums of squares for this data were computed on an Elliott 503 Algol 60 computer using a programme written by Oliver (1968). There was little difference between the fresh weights of heated and unheated plants, but the dry weights of heated plants were far less than those unheated. This was reflected in changes of moisture content; the moisture content of heated plants increased with time of treatment while it decreased in unheated plants as they developed from a vegetative rosette to a flowering form. The dry weight of heated plants increased slightly, but significantly, with time of heat treatment. Spraying with kinetin increased the growth rate of heated plants. The relationship between total nitrogen content and time for the various treatments was similar to that of the first

FIGURE 8.

The moisture contents and dry weights of tobacco plants after varying periods of heat treatment when sprayed daily with either 10^{-4} M kinetin, 10^{-4} M benzyladenine or with water, compared with that of control plants grown in the glasshouse.

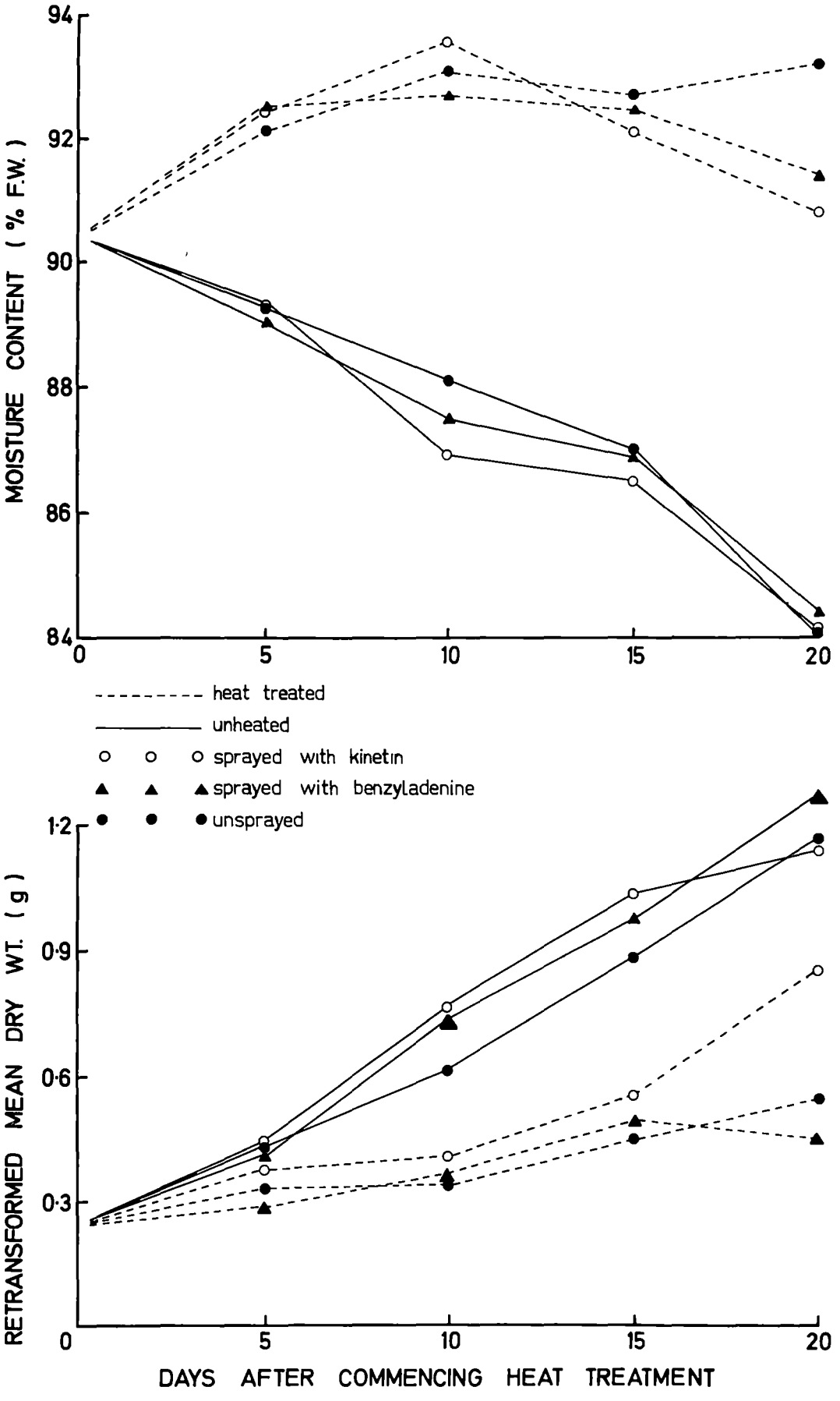
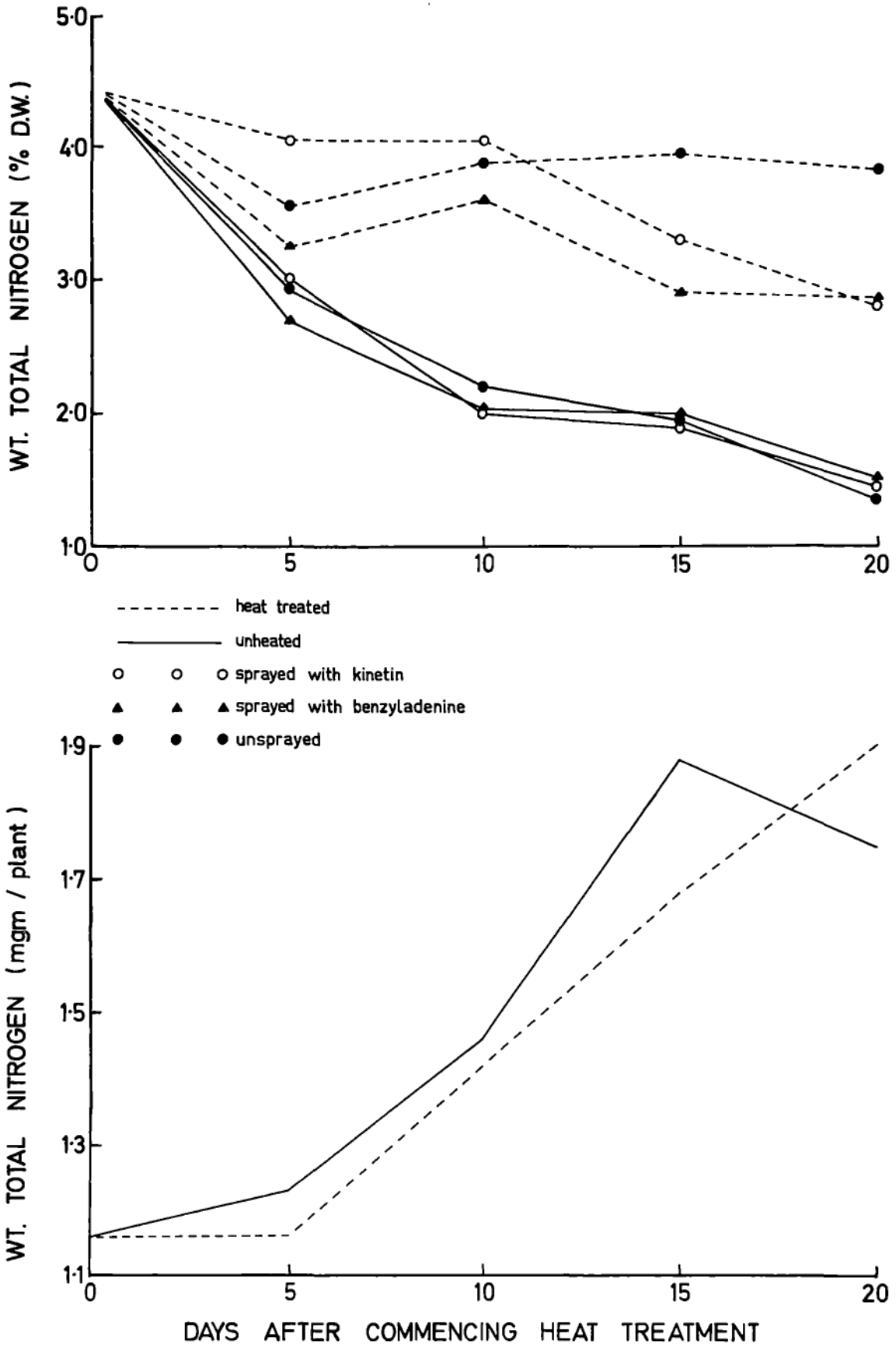

MOISTURE CONTENT (% F.W.)
94
92
90
88
86
84
0
5
10
15
20
heat treated
unheated
sprayed with kinetin
sprayed with benzyladenine
unsprayed
RETRANSFORMED MEAN DRY WT. (g)
1·2
0·9
0·6
0·3
0
5
10
15
20
DAYS AFTER COMMENCING HEAT TREATMENT

FIGURE 9.

The unweighted mean total nitrogen contents (expressed on both a % dry weight basis and an absolute basis) of tobacco plants after varying periods of heat treatment when sprayed daily with either 10^{-4} M kinetin, 10^{-4} M benzyladenine or with water, compared with that of control plants grown in the glasshouse.


WT. TOTAL NITROGEN (% D.W.)
5·0
4·0
3·0
2·0
1·0
0
5
10
15
20
heat treated
unheated
sprayed with kinetin
sprayed with benzyladenine
unsprayed
WT. TOTAL NITROGEN (mgm / plant)
1·9
1·7
1·5
1·3
1·1
0
5
10
15
20
DAYS AFTER COMMENCING HEAT TREATMENT

experiment (see Figure 5). Absolute total nitrogen contents were estimated by multiplying the unweighted dry weight means (i.e. retransformed means) by the unweighted total nitrogen content means. Heated and unheated plants had similar absolute total nitrogen contents and therefore any reduction in the net accumulation of protein in heated plants cannot be attributed to a failure to absorb nitrogenous substrate from the soil medium.

Of necessity, the protein nitrogen analyses were delayed for several months and during this period the dried ground tissue samples were stored in airtight vials in a cool room at $1^{o}C$. The results of the 490 protein nitrogen analyses, when done, were completely farcical. This could not be attributed to analytical technique. It was later learned that the cool room failed to function properly several times during the period of storage, and the meaningless results obtained from the protein nitrogen analyses were presumably due to microbial alteration of the nitrogen forms and losses of nitrogen during these periods.

VII GROWTH OF PLANTS DURING HEAT TREATMENT

The anomaly between the fresh and dry weights of heated and control plants, described in the previous section, prompted further investigations on tobacco plant growth at $36^{o}C$.

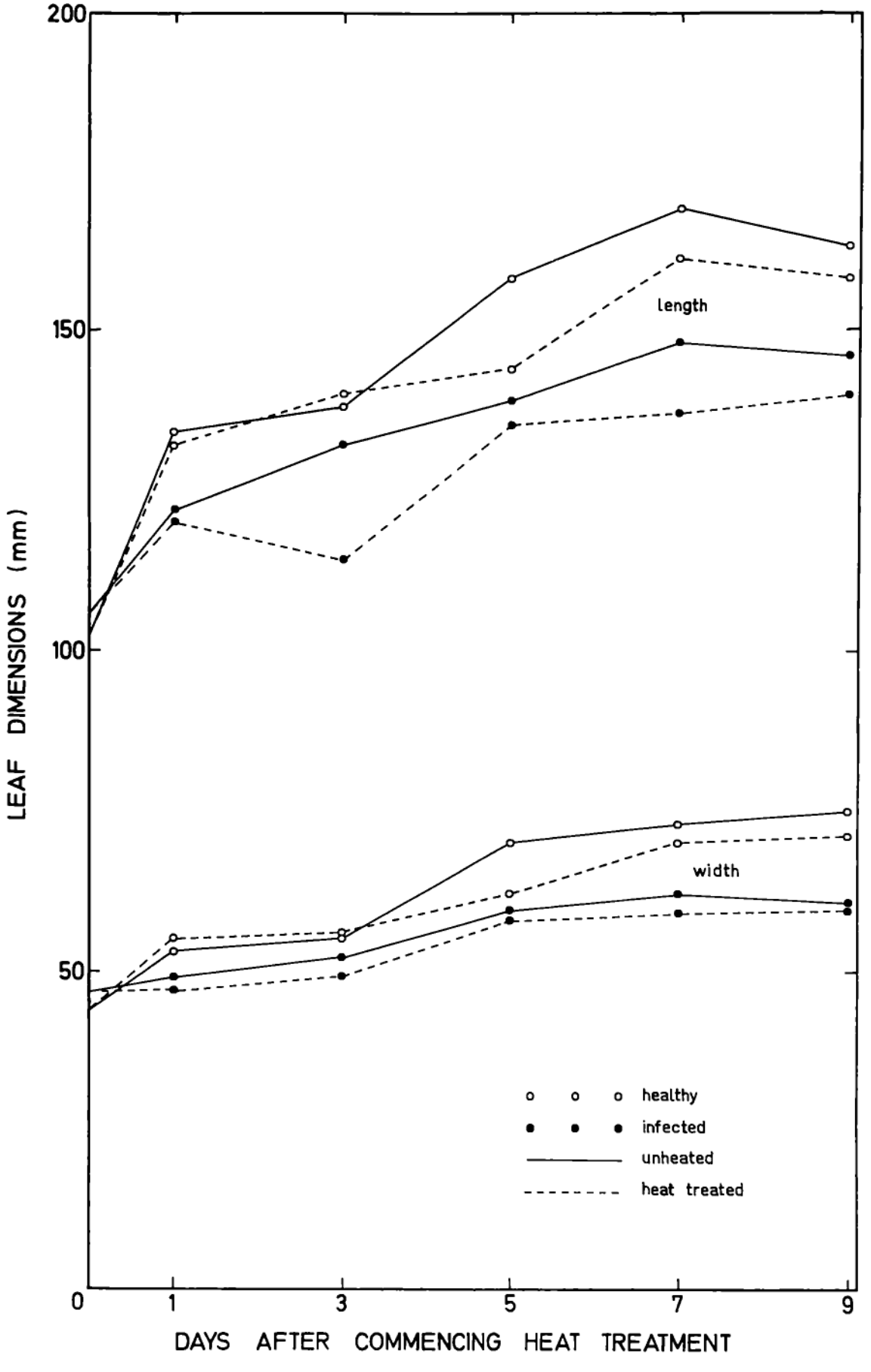
Fifty of a batch of 110 young uniform tobacco plants were inoculated with TAV and held on the glasshouse bench for 14 days. One half of all the plants were then

placed in the phytotron cabinet. Single young leaves about 10 x 5 cm were marked on each plant at this time. These were harvested at random after either 1, 3, 5, 7 or 9 days to measure their length and width. Transverse sections from the central area of some of these leaves were cut on a microtome after fixation with Navashin's fluid, and dehydration and embedding according to the method of Peacock (1966). The sections were stained with safranin and counter-stained with fast green so that measurements could be made of the dimensions of randomly selected palisade mesophyll cells and of the thickness of the leaf sections. The apices from all the plants in this experiment were excised at the time of leaf harvest and fixed immediately in acetic-alcohol. Later, the apical dome was dissected out, together with the first two primordial leaves, and squash-stained in acidified aceto-fuschin (Darlington & LaCour, 1947). Each squash was scanned completely and the meristem given a rating of 0, 1, 2, 3, 4, 5 or 6, according to whether, respectively, 0, 1-5, 6-10, 11-20, 21-50, 50-100 or >100 cells in active division were observed.

There was no significant effect of temperature on leaf size, but the width and length of leaves from infected plants were smaller than those from healthy plants (Figure 10; statistical analyses in Appendix 4). Microscopic examination of leaf sections from plants held at 36°C for 9 days showed that leaf thickness averaged 390 μ compared with 230 μ for the controls. The average dimensions of

FIGURE 10.

Changes in the length and width of leaves on healthy and TAV-infected tobacco plants during heat treatment compared with that of control plants grown in the glasshouse (each point on the graphs represents the mean of the measurements made on the leaves of 5 plants).


200
150
100
50
0
LEAF DIMENSIONS (mm)
length
width
healthy
infected
unheated
heat treated
1
3
5
7
9
DAYS AFTER COMMENCING HEAT TREATMENT

palisade mesophyll cells from heated and unheated plants was 35 x 125 μ and 20 x 80 μ, respectively. The mitotic activity of stem apices remained unaltered in the plants held on the glasshouse bench throughout the experiment. However, mitosis was halted almost completely in plants held at 36°C (Figure 11; statistical analysis in Appendix 5). By the 9th day, the appearance of the meristems from the heat-treated plants was very open due to the continued expansion of cells in leaves which were enclosed within the apex at the commencement of treatment coupled with the failure of the dome to initiate new leaves. There was no effect of infection on mitotic activity.

The change in the ratio of fresh to dry weight of heated plants is due therefore to a stimulation of cell enlargement concomitant with cessation of cell division at 36°C, and this results in the leaves developing a very rugose appearance (Plate 9).

VIII RATE OF PROTEIN SYNTHESIS IN PLANTS DURING HEAT TREATMENT

This section describes experiments designed to find how much the change in protein content of heated plants, described in Section VI, is due to a change in the rate of protein synthesis, and by deduction, an alteration in the rate of protein breakdown.

(a) measurement using $^{35}SO_4^=$

The rates of amino acid and protein synthesis in plants which had been held at 36°C for 5 days were compared with those in similar unheated plants by dipping their ends,

FIGURE 11.

Changes in the mitotic index of the apical meristems excised from tobacco plants after varying periods of heat treatment compared with that of control plants grown in the glasshouse (each point on the graphs represents the mean index recorded for the meristems from 5 healthy and from 5 infected plants).

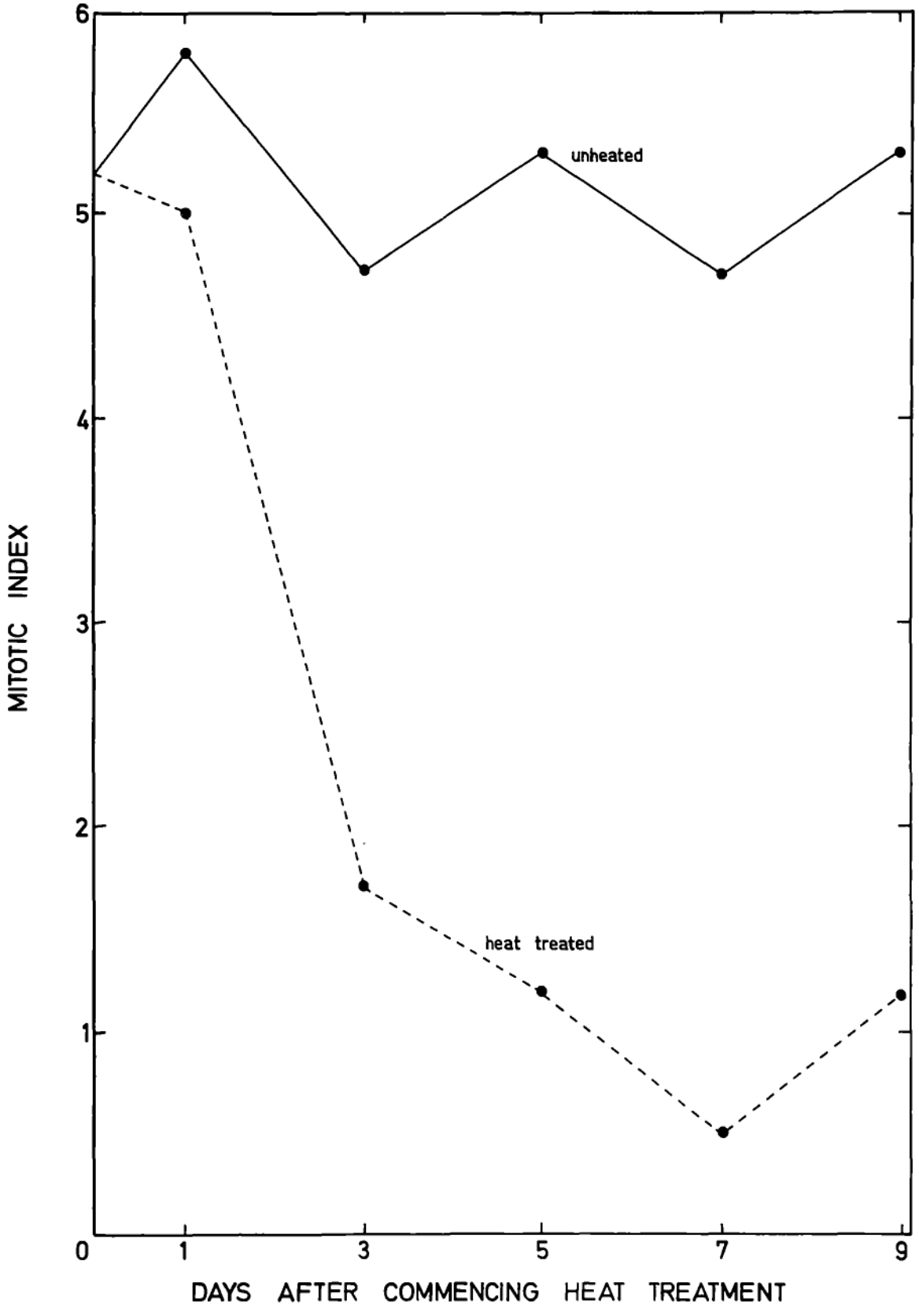

6
5
4
3
2
1
0
MITOTIC INDEX
unheated
heat treated
1
3
5
7
9
DAYS AFTER COMMENCING HEAT TREATMENT

PLATE 9.

A healthy tobacco plant photographed after heat treatment at 36^{o}C for 9 days showing the rugose appearance of the leaves. The dark green colour of the leaves was also typical of heated plants.

which had been cut off at ground level, in glass vials containing 1 mc / ml $^{35}SO_4^=$ (1.16 mc / mM sulphur; Amersham) in 0.02 M Tris-HCl buffer (pH 7.4). Relative humidity about the plants was kept close to 100% to prevent wilting. Incubation in the isotope was for 2 hours to measure incorporation into amino acids and for 6 hours to measure incorporation into protein. The whole experiment was replicated 5 times. After incubation the plants were washed vigorously in tap water to remove externally adsorbed isotope, and then air dried at 65°C. Weighed aliquots of the ground, dried leaf tissue were wrapped in filter paper, and non-incorporated isotope extracted by boiling for 2 hours in 5% trichloroacetic acid (containing 10% sodium sulphate) and then successively extracted in Soxhlet apparatus for 2 hours with water, ethanol and ether. The residual protein was resuspended by heating in formamide at 90°C for 24 hours. Radioactivity of the samples was measured in a Unilux liquid scintillation counter in glass vials containing 15 ml of the diotol solution used by Herberg (1960). The counts were corrected for background radiation, efficiency of the counter, and for quenching by adding carrier-free $^{35}SO_4^=$ in aqueous solution. Amino acids in the trichloroacetic acid extract were separated by descending chromatography in two directions on Whatman no. 1 filter paper after oxidation with performic acid (Moore, (1963). The first solvent used was butanol:acetic acid: water, and the second was water saturated phenol. Amino

acid spots shown up by ninhydrin were tested for radioactivity with a geiger counter.

No radioactivity was detected in any of the amino acids extracted from the plants and separated by two dimensional paper chromatography. The rate of protein synthesis in heated plants was about 50% of that in unheated plants (Table 24). An oversight in this experiment was that the total amount of isotope absorbed by the two groups of plants was not measured and therefore, because plants at the higher temperature generally absorb more isotope (see following section), the figure presented in Table 24 for the heated plants is probably an inflated estimate. However, irrespective of this, the change in absolute plant protein content during heat treatment (Figure 7), on the basis of the results in Table 24, must be due to both an increased rate of protein degradation as well as a decreased rate of synthesis.

(b) validity of the method of measurement

Two aspects of procedure adopted in the above experiment may have produced invalid results. Firstly, the amount of $^{35}SO_4^{=}$ label incorporated into protein was small and variable, as evidenced by the low level of significance between the apparently large difference recorded in the average rates of protein synthesis between the two environments. Secondly, the rate of protein synthesis in detached tobacco cuttings may be different from that in intact plants, and that these possible

TABLE 24.

The incorporation of $^{35}SO_4^{=}$ into the proteins of heated and unheated plants over a 6-hour incubation period.

Treatment	Mean cpm/g (fresh wt.)
unheated	1234*
heated	622*
% reduction	49.6
level of significance	$P < 0.05$

* figures represent the means for five separate plants in each instance.

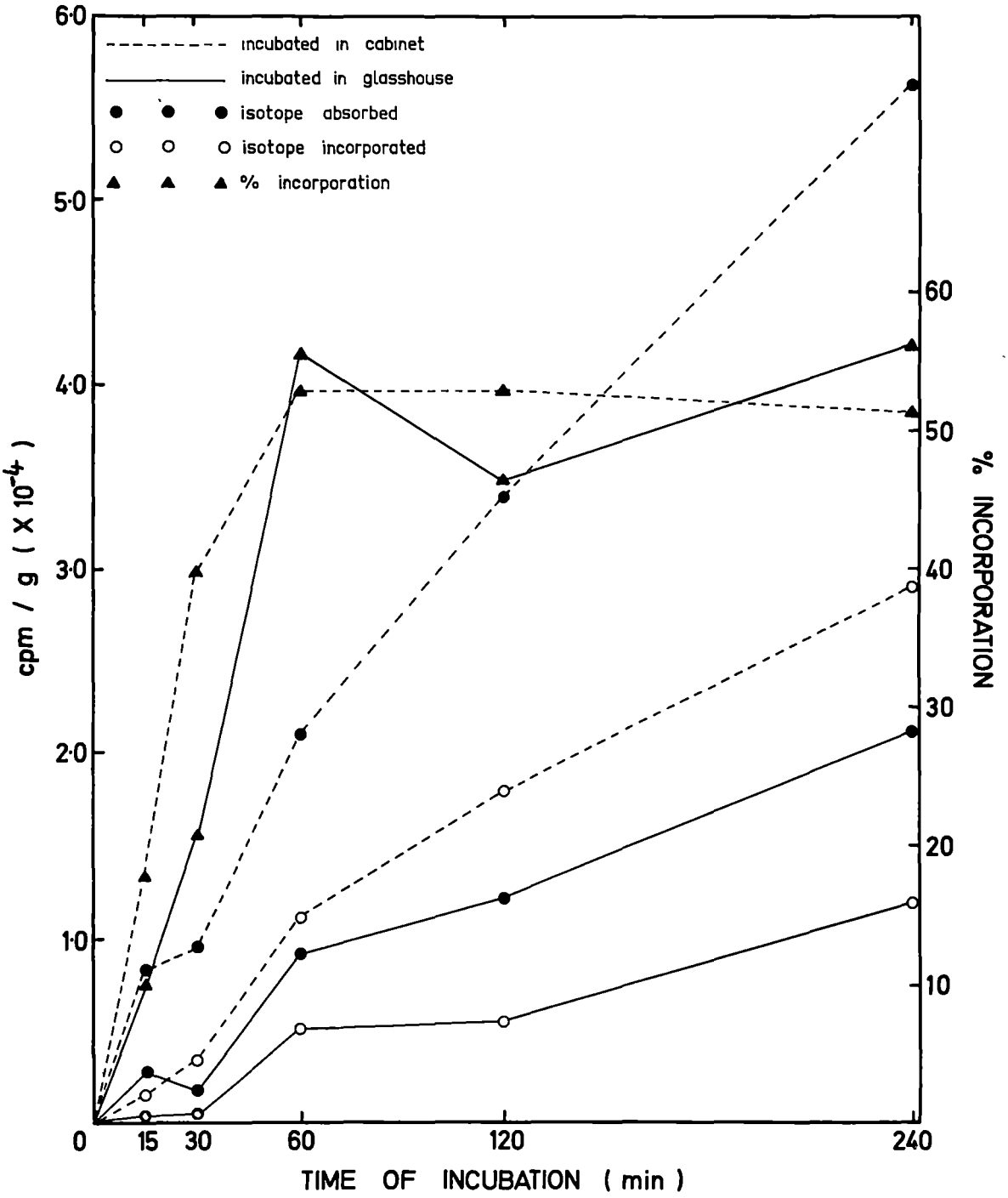
differences would become accentuated with time following excision.

Therefore, the rates of protein synthesis were measured in excised cuttings from healthy unheated plants incubated in vials on the glasshouse bench containing 1 mc/ml of Chlorella ^{14}C protein hydrolysate (52 mc/m atom carbon; Amersham) for periods of from 15 to 240 minutes. At the same time, measurements were made on similar plants, which had been heat-treated at 36°C for 12 days, incubated in the isotope in the phytotron cabinet. Non-incorporated amino acids were extracted from the dried tissue samples as described in the previous section, except that sodium sulphate was omitted from the 5% trichloroacetic acid extractant. The residual protein was hydrolysed by boiling in 6 N HCl for 18 hours in a flask fitted with a reflux condenser. Samples of the hydrolysate were dried under vacuum at 40°C, and then twice washed with water and dried before suspending in scintillant. Unextracted tissue samples were treated in the same way so that total ^{14}C uptake could be determined. ^{14}C-hexadecane was used to estimate the degree of quenching in each sample.

The results, presented in Figure 12, show that the amount of isotope absorbed was almost linear with time of incubation in both environments. The % of absorbed isotope incorporated into protein increased with time of incubation up to 60 minutes and then remained constant. With shorter periods of time an unsteady state existed,

FIGURE 12.

The absorption of ^{14}C Chlorella protein hydrolysate over periods of from 15 to 240 minutes, by excised, unheated tobacco plants incubated in vials on the glasshouse bench compared with that of plants which had been heat treated for 12 days incubated in vials in the phytotron cabinet, together with the amount and proportion of the absorbed isotope incorporated into protein by the two groups of plants.


incubated in cabinet
incubated in glasshouse
isotope absorbed
isotope incorporated
% incorporation
cpm / g (X 10⁻⁴)
% INCORPORATION
TIME OF INCUBATION (min)
0
15
30
60
120
240
1·0
2·0
3·0
4·0
5·0
6·0
10
20
30
40
50
60

probably due to absorbed isotope being confined chiefly to the conducting elements. After one hour, there was no difference between the % incorporation in plants incubated at high or low temperatures even although the absolute amounts taken up varied greatly.

The efficiency of the successive extraction procedures in removing absorbed non-incorporated isotope was also checked during this experiment. The mean activities of the extractants used to remove non-incorporated isotope are set out in Table 25. These show that all absorbed isotope was accounted for, and the activities of the successive extractants suggest that there would have been negligible amounts of non-incorporated isotope present in the final residue.

In a subsidiary experiment done at the same time, measurements were made on intact plants of about the same size and stage of development which were growing in nutrient solution culture in the two environments. Isotope was added to the nutrient solutions (6 μC ^{14}C/ml of Chlorella protein hydrolysate in 300 ml of nutrient solution per plant) and the amounts of absorbed and incorporated isotope were determined after 8 hours incubation. This experiment was replicated three times, and the results paralleled those recorded in detached shoots (Table 26).

The above results indicate that the rates of protein synthesis measured in detached shoots over short periods of time following excision appear to reflect the

TABLE 25.

Mean cpm / g of successive extracts during the isolation of leaf proteins.

Extract	Mean cpm / g (fresh weight)
trichloroacetic acid	3,924
water	1,248
ethanol	149
ether	7
residual (incorporated into protein)	3,648
combined total of extracts	8,976
measured total uptake	8,763

TABLE 26.

Incorporation of radioactivity during an 8-hour period at 25° and 36°C into the leaf proteins of intact plants growing in nutrient solution containing ^{14}C *Chlorella* protein hydrolysate.

Parameter	Incubation Temperature	
	25°C	36°C
mean isotope uptake (cpm / g)*	155,300	55,792
mean isotope incorporation (cpm / g)*	91,233	30,125
mean % incorporation	57.6	53.9
standard error of %	7.2	1.6

* figures represent the mean values for 3 plants.

natural 'in vivo' situation. This was very convenient, as the use of detached shoots in preference to intact plants growing in culture solution offers several technical advantages.

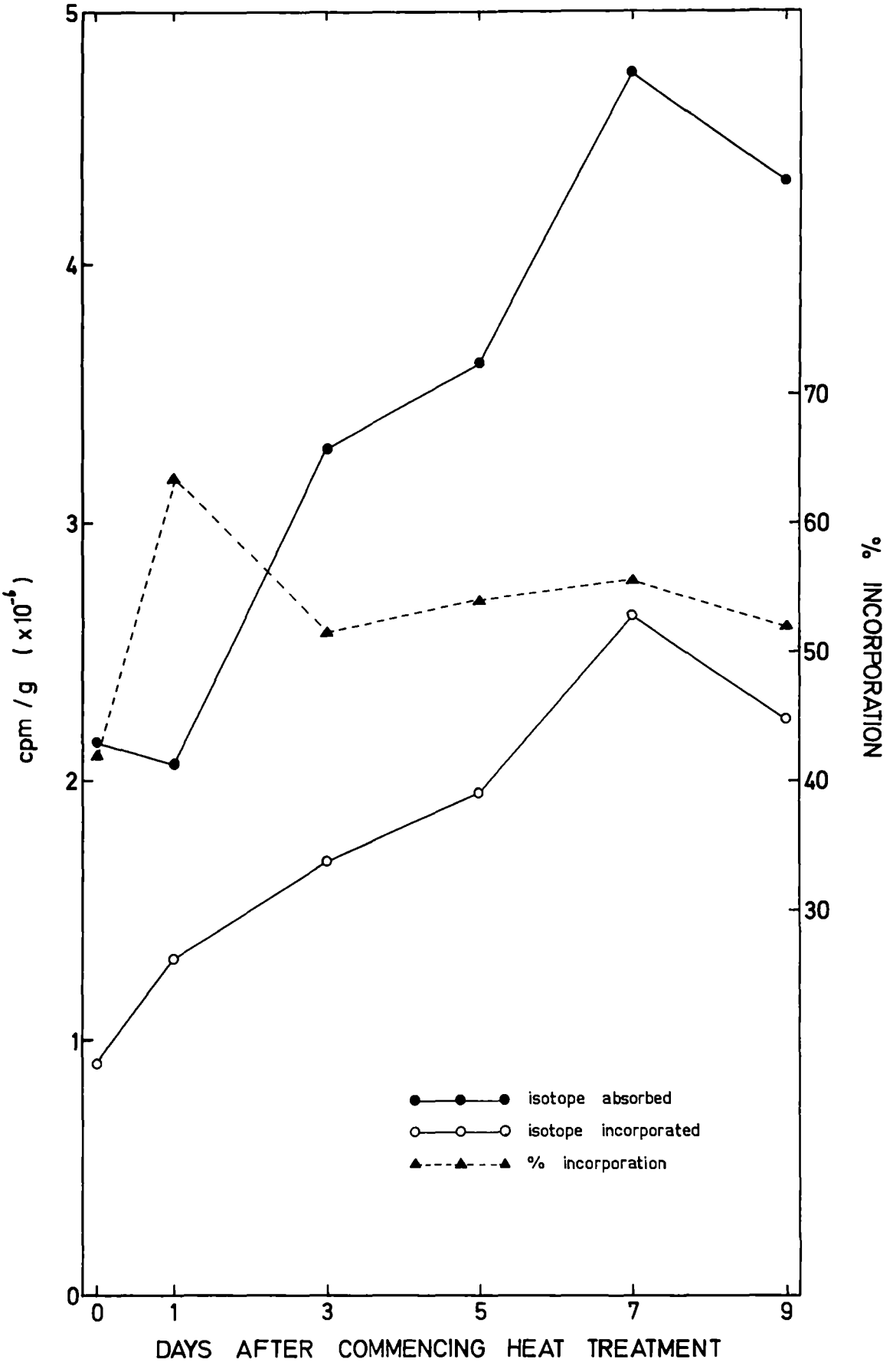
(c) effect of time of heating

Incorporation of ^{14}C *Chlorella* protein hydrolysate into protein was measured in excised plants in the phytotron cabinet over a two-hour period. These plants had been heated beforehand at 36°C for either 0, 1, 3, 5, 7 or 9 days. Individual plants for each treatment were placed in the cabinet on different days so that the actual uptakes could be done simultaneously. The experiment was repeated on five successive days with single plants which had been heated for each of the six periods of time. Measurement of isotope absorption and incorporation were done on bulked samples of five plants due to the lengthy extraction procedure and because data from previous experiments had shown that the deviation between samples within treatments was always less than 5%.

There was no difference between the rate of incorporation by plants heated for 3, 5, 7 or 9 days; this rate was much higher than that in unheated plants, but less than that in plants heated at 36°C for 1 day (Figure 13). Thus, in contradiction to the $^{35}SO_4^{=}$ study where heating for 5 days was estimated to reduce the rate of protein synthesis by about 50%, these results, using a protein hydrolysate as substrate, suggest that the rate of protein

FIGURE 13.

The absorption of ^{14}C *Chlorella* protein hydrolysate by shoots excised from tobacco plants which had been heated for varying periods of time, together with the amount and proportion of the absorbed isotope incorporated into protein.


5
4
3
2
1
0
cpm / g (x10⁻⁶)
70
60
50
40
30
% INCORPORATION
0
1
3
5
7
9
DAYS AFTER COMMENCING HEAT TREATMENT
isotope absorbed
isotope incorporated
% incorporation

synthesis increases in heat-treated plants.

IX RIBOSOME CONCENTRATION IN PLANTS DURING HEAT TREATMENT

The studies reported in this section were done in a Spinco model E analytical ultracentrifuge. Samples were examined in a 12 mm kel-F cell with a 2^{o} sector and spun in an An-D rotor fitted with a counter balance. Operational speeds used were 24,630, 35,600 and 57,980 r.p.m., and temperature was controlled at $2^{o}C$. Exposures were taken on Kodak metallographic plates using Schlieren optics and observed s values, calculated by the graphical method of Markham (1960), were corrected to $s_{20,w}$ by reference to the observed s value of fraction I protein or that of added purified tobacco mosaic virus, or both. This was necessary because of the difficulties encountered when attempting to measure the viscosity of plant extracts accurately.

(a) preliminary experiments

The leaves from 10 healthy, unheated plants were ground in the cold without any diluent and squeezed through cheesecloth to express the sap. The homogenate was clarified by low speed centrifugation and then examined in the analytical ultracentrifuge. A similar group of 10 plants which had been heated beforehand at $36^{o}C$ for 5 days were treated in the same way. This procedure was then repeated with batches of plants which had been infected with TAV 17 days previously except that the heated plants had been treated for only 3 days.

Comparable exposures of the Schlieren diagrams for the healthy plant extracts are illustrated in Plate 10, and those for the infected plants in Plate 11. Heat treatment reduced the concentration, as measured by peak area, of the four major components observed in the Schlieren diagrams, viz. the cytoplasmic ribosomes (80 s), the chloroplast ribosomes (70 s), fraction I protein (about 18 s) and the smaller cytoplasmic proteins (fraction II protein). Reductions in peak area after heating were greater for the chloroplast components than for those from the cytoplasm (Table 27). There were no differences between the sedimentation coefficients of the ribosome peaks from heated and unheated plants. However, fraction I protein appeared to sediment faster and fraction II protein slower than in the control unheated plant extracts. Apparently, virus concentration was too low to produce a peak in the infected plant extracts.

(b) <u>significance of peak area and s value</u>

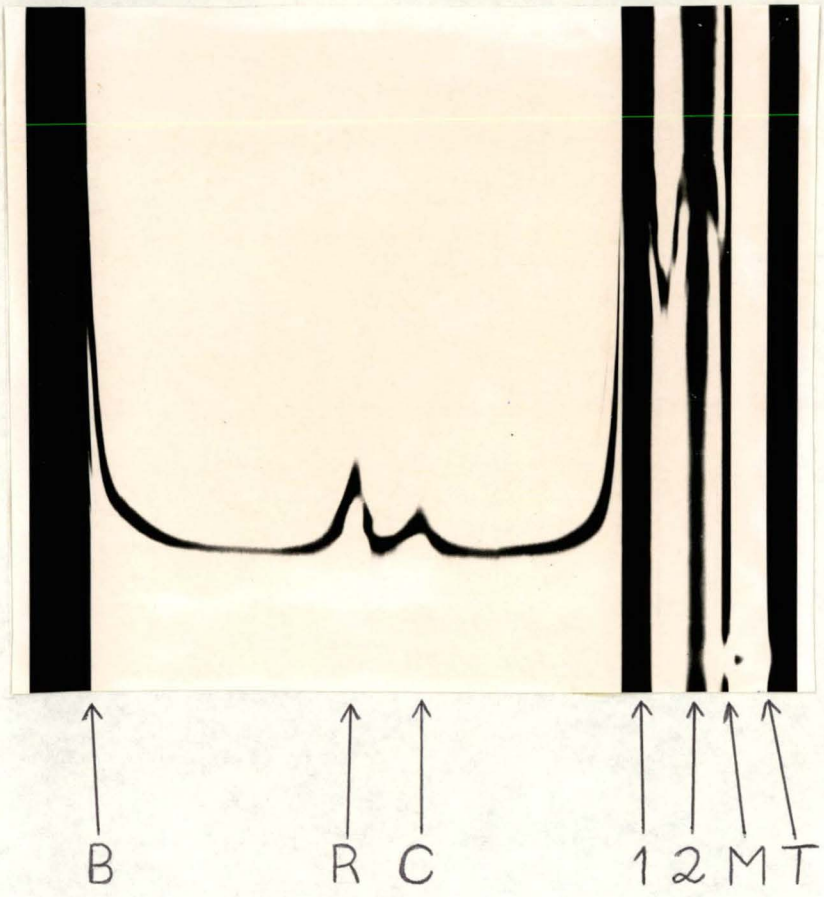
Cytoplasmic ribosomes were purified from dark-grown pea epicotyls, by the method of Bonner (1965), using two cycles of high and low speed centrifugation. Dilutions of this preparation were then made with magnesium-containing buffer and these were examined in the analytical ultracentrifuge. Peak areas were measured from enlargements of exposures taken eight minutes after reaching speed (35,600 r.p.m.).

The Schlieren diagrams showed that the method of

PLATE 10

Schlieren diagrams of sap extracts from healthy tobacco plants photographed 16 minutes after reaching speed at 35,600 rpm with a bar angle of 55°. The upper diagram was that produced by the extract from unheated plants, and the lower diagram was that produced by the extract from plants which had been heated at $36^{\circ}C$ for 5 days.

(T = top of cell, M = meniscus, 2 = fraction II protein, 1 = fraction I protein, C = chloroplast ribosomes, R = cytoplasmic ribosomes, D = ribosome dimers, B = bottom of cell).


B
R
C
1
2
M
T

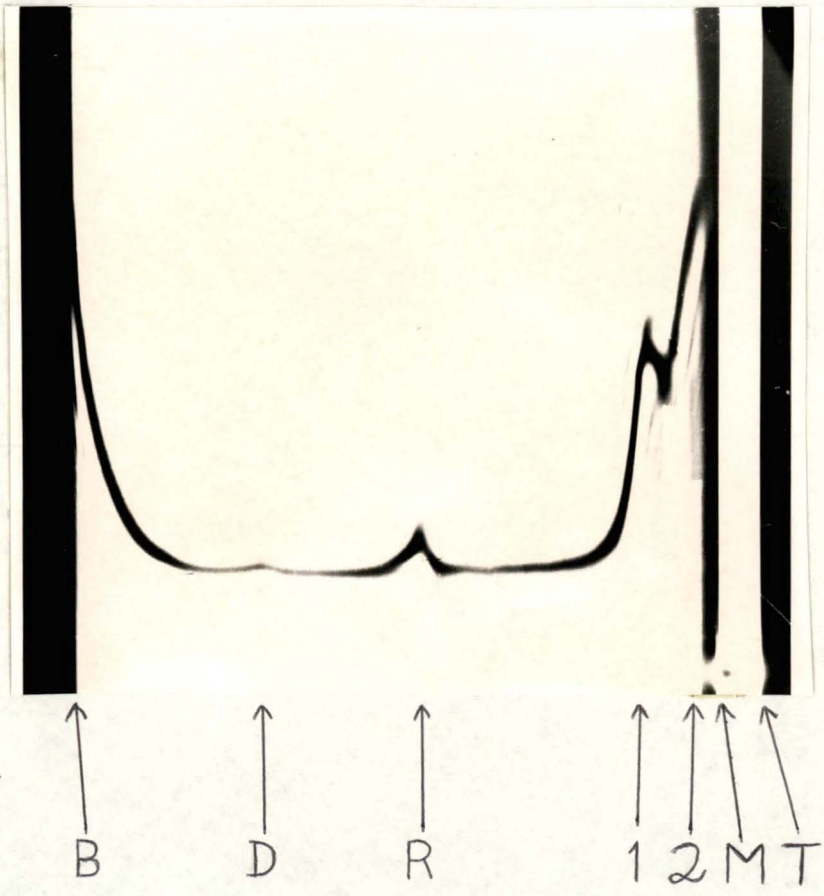
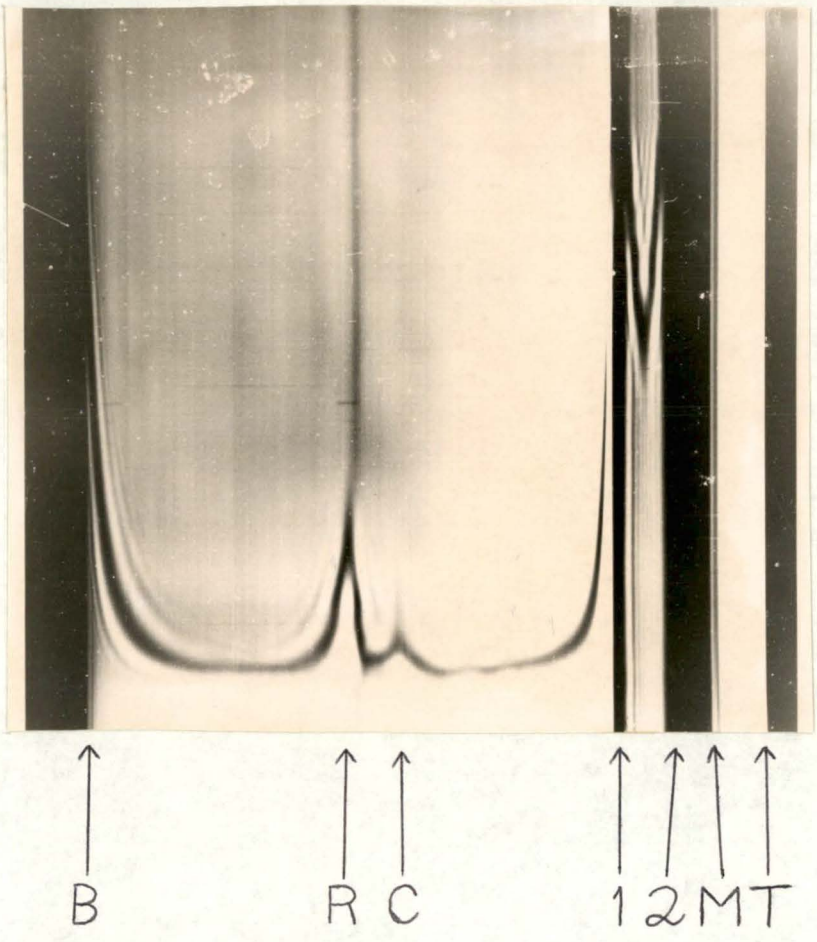

B
D
R
1
2
M
T

PLATE 11.

Schlieren diagrams of sap extracts from infected tobacco plants photographed 16 minutes after reaching speed at 35,600 rpm with a bar angle of 55°. The upper diagram was that produced by the extract from unheated tobacco plants, and the lower diagram was that produced by the extract from plants which had been heated at 36°C for 3 days.

(T = top of cell, M = meniscus, 2 = fraction II protein, 1 = fraction I protein, C = chloroplast ribosomes, R = cytoplasmic ribosomes, B = bottom of cell).


B
R C
1 2 M T

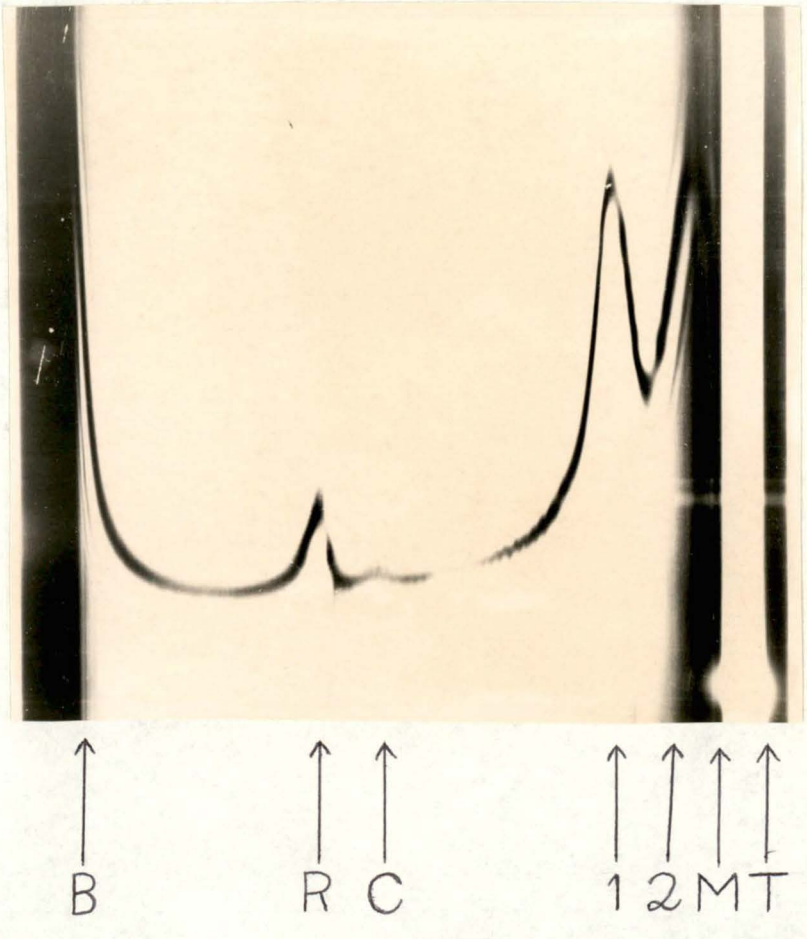

B
R C
1 2 M T

TABLE 27.

Relative peak areas on Schlieren diagrams of cell components in sap extracts from healthy and virus-infected plants heated for varying periods of time.

Infection	Treatment	Relative Peak Areas of Cell Components			
		cytoplasmic ribosomes	chloroplast ribosomes	fraction I protein	fraction II protein
healthy	unheated	100	100	100	100
	36°C / 5 days	24.6	2.4	34.2	85.0
infected	unheated	100	100	100	100
	36°C / 3 days	51.2	15.0	66.1	74.2

purification yielded very pure ribosome preparations (Plate 12). Values of the measured peak areas and calculated sedimentation coefficients for the various dilutions of the ribosome preparation are set out in Table 28. These indicate that, as might be expected from theoretical considerations (Schachman, 1959), peak area is directly proportional to ribosome concentration and that the apparent sedimentation coefficient increases with dilution. The measured peak area of any one preparation decreased with increasing time of centrifugation due to the effect of radial dilution. However, measurements of peak areas in different centrifuge runs were always comparable if taken at similar times after reaching operational speed. It was easier to measure peak areas accurately after shorter rather than longer periods of centrifugation.

(c) comparison of extraction procedures

The undiluted sap extracts examined in the preliminary experiment were very dark, particularly those from heated plants, which necessitated long exposure times resulting in blurred prints. Also, polymerisation of oxidised phenolic compounds in the sap resulted in a marked curvature of the Schlieren baseline.

Two possible methods of overcoming this problem were examined. Firstly, some of the polyphenoloxidase present in the sap extract was removed by clarification with 0.10 volumes of chloroform and secondly, the leaf tissue was triturated with an equal weight of chromatographic grade

PLATE 12.

Schlieren diagram of a cytoplasmic ribosome preparation purified from pea epicotyls photographed 12 minutes after reaching speed at 35,600 rpm with a bar angle of 55°.

(T = top of cell, M = meniscus, R = cytoplasmic ribosomes, D = ribosome dimers, and B = bottom of cell).

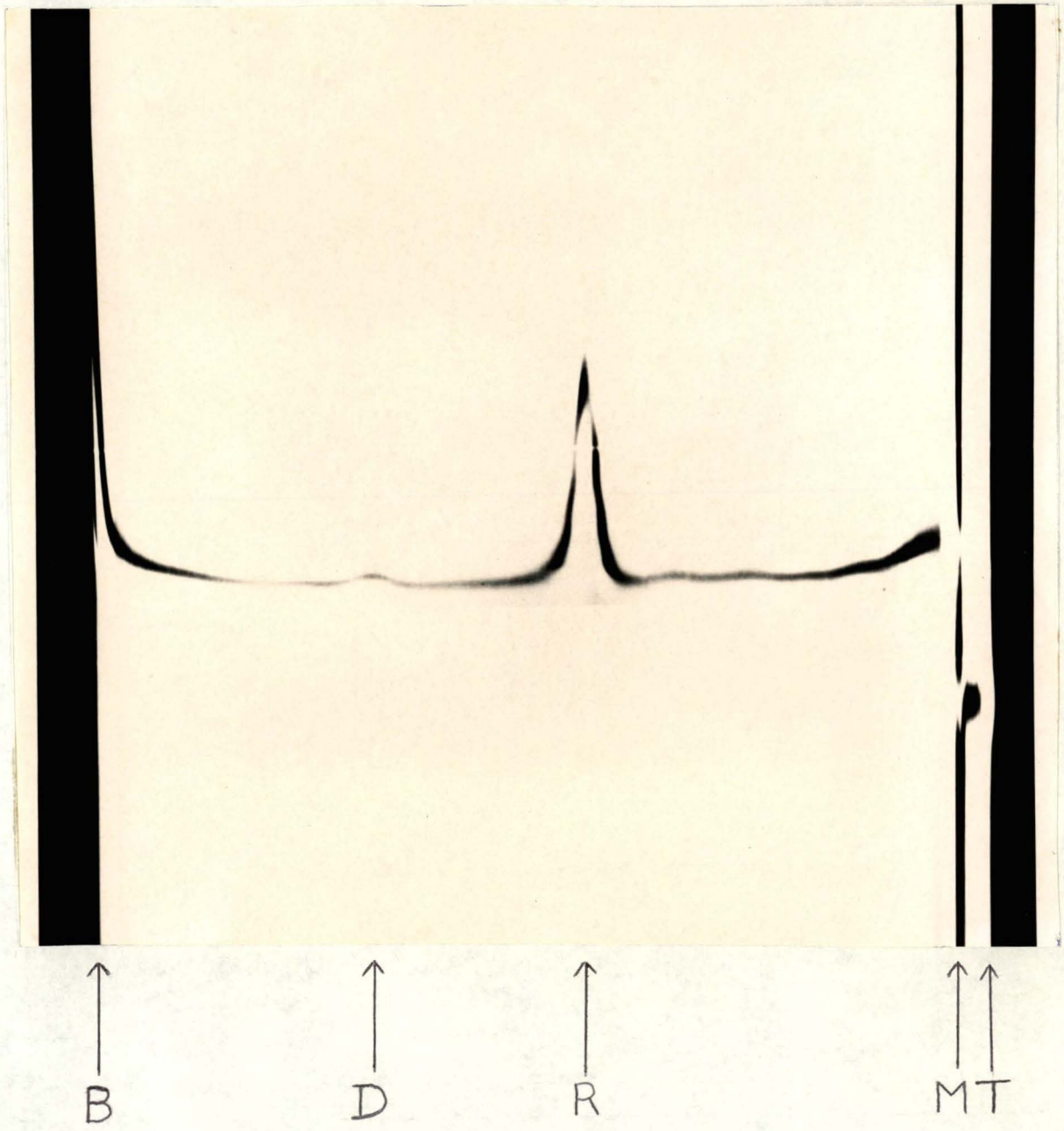

B
D
R
M
T

TABLE 28.

Relative peak areas and calculated sedimentation coefficients of dilutions of a purified pea cytoplasmic ribosome preparation.

Relative Concentration	Relative Peak Area	Sedimentation Coefficient*
1.00	1.00	63
0.75	0.73	65
0.50	0.41	67
0.25	0.28	69

* at $2^{o}C$ in $5X10^{-3}$ M Tris-HCl (pH 7.8) containing 10^{-3} M $MgCl_2$.

alumina to adsorb the phenolic compounds from the expressed sap.

Alumina was very effective in producing light green sap extracts. The chloroform extract was intermediate in colour between the alumina and untreated extracts. The Schlieren patterns produced by these three preparations were also markedly different (Plate 13).

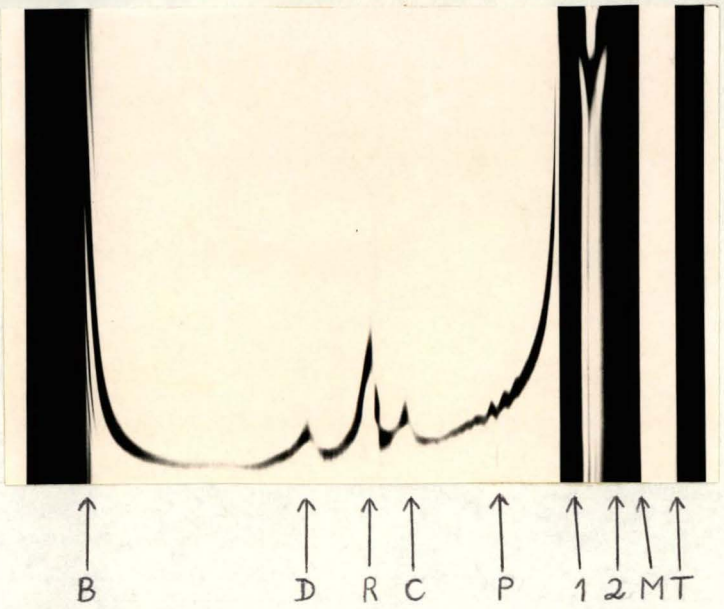
The concentration of chloroplast (70 s) ribosomes was much greater in the treated extracts. Presumably, many of the chloroplast membranes are not ruptured by the normal trituration procedure. However, in the alumina extract, many of the chloroplast ribosomes released were broken into their sub-unit components due to adsorption of magnesium ions from the cell sap. This provides some alternative additional evidence for the greater magnesium requirement of 70 s chloroplast ribosomes for stability compared with those of 80 s from the cytoplasm (Boardman et al., 1966).

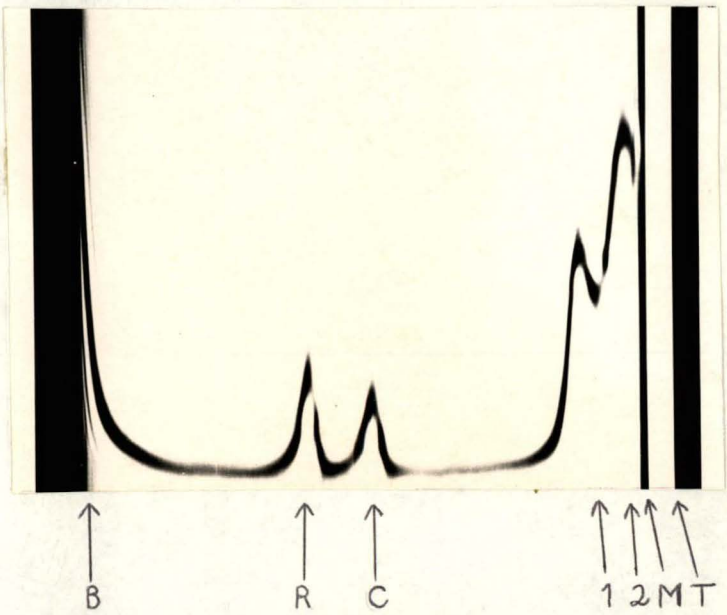
The concentration of cytoplasmic ribosomes was similar in the control and chloroform treated extracts, although chloroform had the advantage in that it separated some dimers present in the untreated extract into their monomeric units. Alumina apparently adsorbed some of the 80 s particles from the sap extract. Alternatively, it may be that alumina, but not chloroform, selectively ruptured or solubilised the membranes of some cellular organelle releasing substances which destroy cytoplasmic ribosomes, because a test on the possible use of sodium

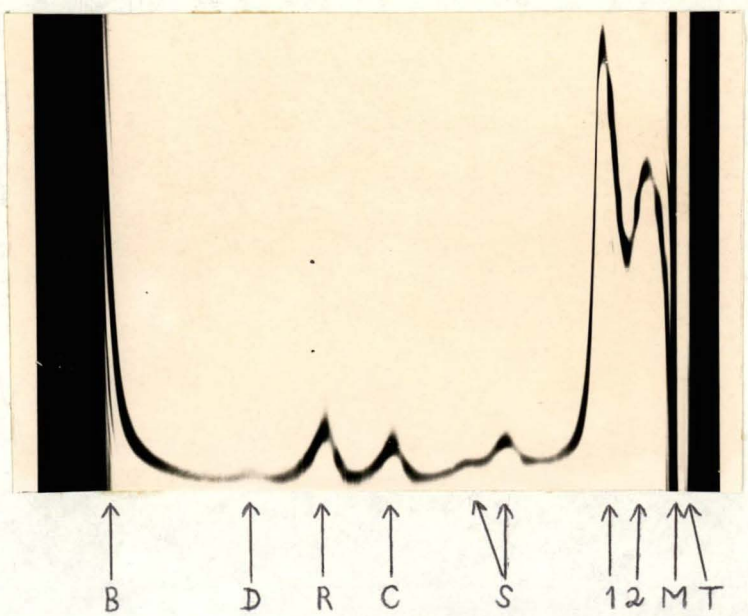
PLATE 13.

Schlieren diagrams of sap extracted from healthy tobacco leaves photographed 20 minutes after reaching speed at 35,600 rpm with a bar angle of 55^{o}. The upper diagram was that produced by an untreated extract, the central diagram was that produced after clarification of the sap with 0.10 volumes of chloroform, and the lower diagram was that produced by sap extracted from leaves in the presence of alumina.

(T = top of cell, M = meniscus, 2 = fraction II protein, 1 = fraction I protein, P = polymerised oxidised phenolic compounds, S = sub-unit components of chloroplast ribosomes, C = chloroplast ribosomes, R = cytoplasmic ribosomes, D = ribosome dimers, and B = bottom of cell).


B
D
R
C
P
1
2
M
T


B
R
C
1
2
M
T


B
D
R
C
S
1
2
M
T

deoxycholate in a previous experiment to release bound ribosomes, through solubilising all intracellular membranes, yielded no ribosomal particles whatever. Sodium deoxycholate is frequently used in the preparation of ribosomes from animal cells to free those attached to endoplasmic reticulum (Bonner, 1965).

Alumina and chloroform, respectively, adsorbed and denatured considerable amounts of fraction I and II proteins present in the untreated extract, and they were differentially selective in the proportions of the two fractions which they removed.

The baselines of the Schlieren diagrams of the chloroform and alumina extracts were practically horizontal while that of the control extract showed marked curvature. This was due to the polymerisation of oxidised phenolics in the control extract which resulted in a continuum of peaks of decreasing amplitude sedimenting at values between 18 s and 70 s. The formation and presence of these polymers in the extract had no effect on the area of the peaks (Plate 14).

(d) changes with time of heat treatment

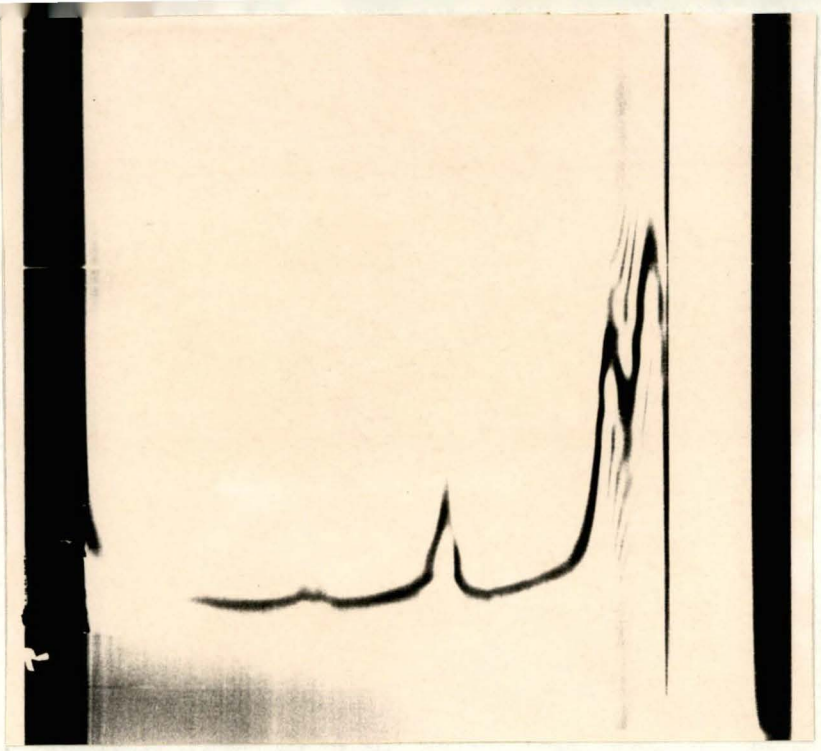
The relative concentrations were measured of the ribosome and major protein components in extracts from plants which had been heated at 36^{o}C for 0, 1, 3, 5, 7 and 9 days. The plants used were an extremely uniform lot and a bulked sample of 10 plants was used to produce each extract. They were placed in the cabinet at various times so that the extracts could be examined simultaneously. Tissue was

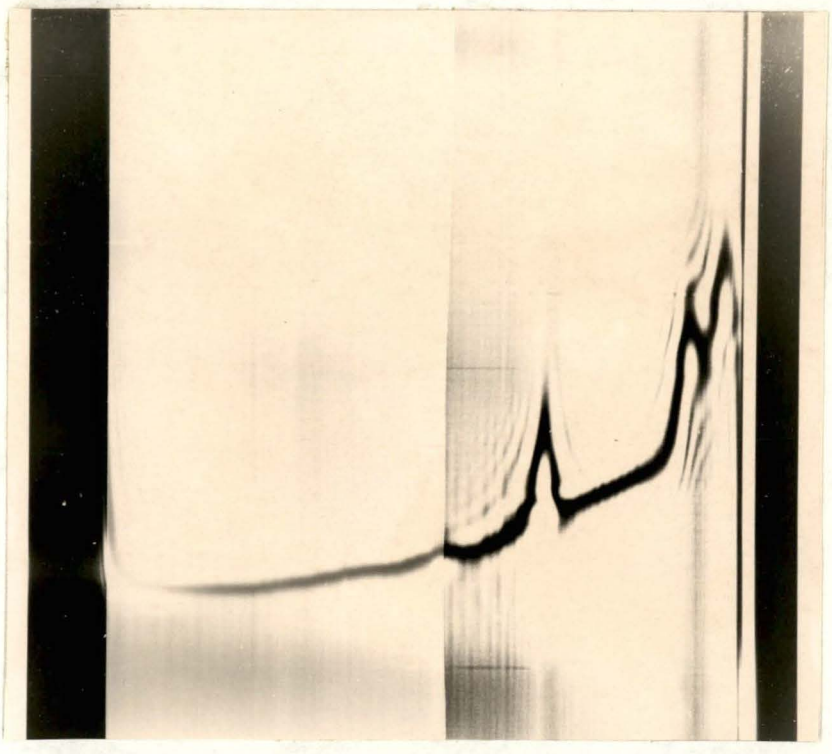
PLATE 14.

Schlieren diagrams of sap* extracted from healthy tobacco leaves photographed 16 minutes after reaching speed at 35,600 rpm with a bar angle of 50^{o}. The upper diagram was that produced by an aliquot of the sap examined immediately after extraction, and the lower diagram was that produced by an aliquot which had been held at $0^{o}C$ for 8 hours following extraction prior to examination in the ultracentrifuge.

(T = top of cell, M = meniscus, 2 = fraction II protein, 1 = fraction I protein, P = polymerised oxidised phenolic compounds, R = cytoplasmic ribosomes, D = ribosome dimers, and B = bottom of cell).

* The tissue was extracted (1:1, W:V) in 0.01 M Tris-HCl buffer containing 10^{-3} M Mg^{++} (pH 7.0).


B
D
R
12M
T


B
D
R
12MT

extracted (1:1, W:V) in 0.01 M Tris-HCl buffer containing 10^{-3} M Mg^{++} (pH 7.0) as chloroplast structural protein may be unstable in extracts of low pH (Francki, private communication), and the pH of plant sap extracts was known to decrease with time of heat treatment (see section XVII). The extracts were filtered through cheesecloth and clarified with 0.10 volumes of chloroform prior to examination in the analytical ultracentrifuge.

Comparable Schlieren diagrams of each extract are shown in Plate 15, and the relative peak areas of the cytoplasmic ribosomes are set out in Table 29. The concentration of cytoplasmic ribosomes (80 s) rose slightly during the first three days of heat treatment, and then declined rapidly to a low level. There was no simple relationship between time of heating and 80 s ribosome concentration. The extracts contained no detectable concentration of chloroplast ribosomes, although chloroplast structural protein was readily detected. Accurate measurement of the areas of the protein components was not possible due to variations in the curvature of the baseline between extracts. However, the same trends were apparent for these peaks as those depicted in Plates 10 and 11.

X CONCENTRATION OF RNA FRACTIONS IN PLANTS DURING HEAT TREATMENT

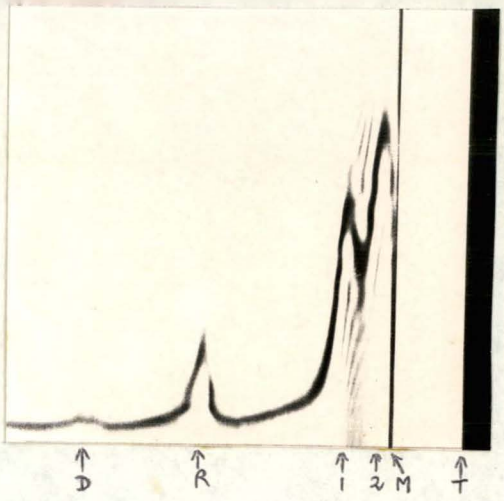
Four attempts were made to extract the ribosomal, viral, messenger and transfer RNA fractions from heated and unheated plants using diethylcarbonate as an inhibitor of ribonuclease (Solymosy et al., 1968). Final separation of

PLATE 15

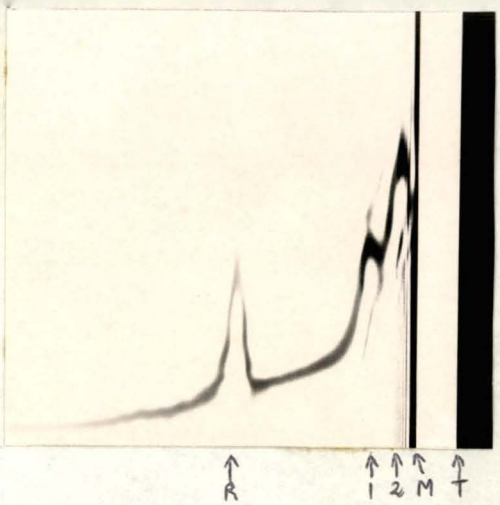
Schlieren diagrams of sap* extracted from healthy tobacco plants, which had been heated at 36°C for varying periods of time, photographed 16 minutes after reaching speed at 35,600 rpm with a bar angle of 50°.

(T = top of cell, M = meniscus, 2 = fraction II protein, 1 = fraction I protein, R = cytoplasmic ribosomes)

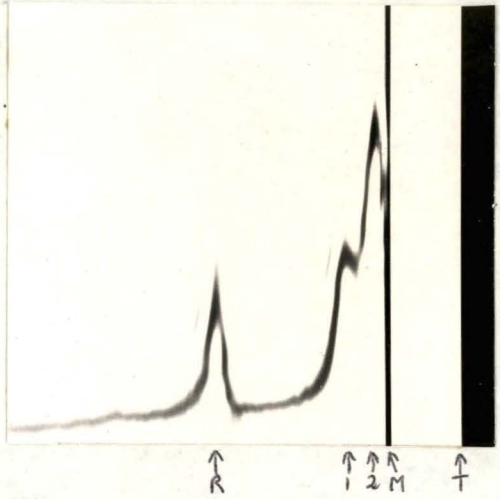
* The tissue was extracted (1:1, W:V) in 0.01 M Tris-HCl buffer containing 10^{-3} M Mg^{++} (pH 7.0).



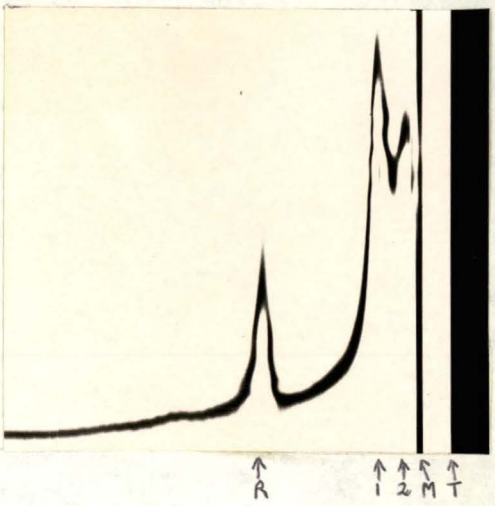
DAY 0



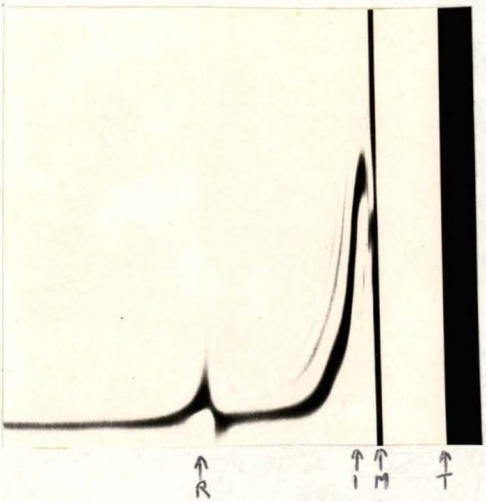
DAY 1



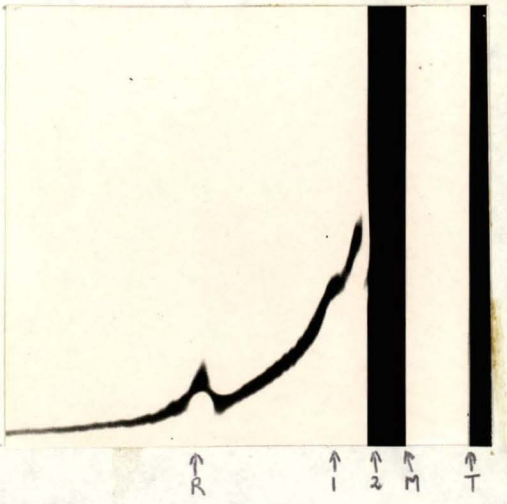
DAY 3



DAY 5



DAY 7



DAY 9

TABLE 29.

Relative peak areas of cytoplasmic ribosomes in extracts from tobacco plants heated at 36°C for varying periods of time.

Length of Treatment (days)	Relative Peak Area
0	1.00
1	1.13
3	1.35
5	0.96
7	0.26
9	0.22

the RNA fractions was attempted both on RNase-free sucrose density gradients and in the analytical ultracentrifuge.

Although every precaution was taken to guard against ribonuclease contamination by autoclaving all glassware, etc., the final products contained only low molecular weight heterogeneous material which remained near the meniscus during centrifugation (Figure 14).

Later experiments indicated that my source of diethylcarbonate would not inhibit RNase infectivity as the chemical did not increase the infectivity of inocula prepared from tobacco leaves in its presence (results set out in Table 46b).

XI COMPETITION BETWEEN TAV AND PLANT PROTEIN SYNTHESIS

(a) actinomycin D in culture solutions

Tobacco plants were raised in nutrient culture solutions in the glasshouse. Upon reaching sufficient size, some of them were inoculated with TAV and simultaneously actinomycin D was added to half of the solutions to a final concentration of 1 µg / ml.

Symptoms of TAV infection 14 days later were more intense on plants grown in the presence of the antibiotic and this was correlated with the virus concentration measured in both the leaves and the roots (Table 30). There appeared to be less polyphenoloxidase activity in the extracts from plants grown in the presence of actinomycin D during preparation of the inocula. Also, healthy plants developed symptoms resembling those of TAV infection when

FIGURE 14.

The optical densities of fractions* collected from sucrose density gradients which had been layered with RNA preparations extracted from tobacco plants according to the method of Solymosy et al. (1968).

* collected by puncturing the bottom of tubes after centrifugation for 6 hours in a 25.1 SW rotor at 25,000 rpm; each fraction contained approximately 10 drops of solution, and was weighed and diluted according to volume so that they were directly comparable; variations in the volume of fractions resulted in varying numbers of fractions being collected from the three tubes; the optical densities of only every 5th fraction is plotted as there were no fluctuations in the absorbance of the intermediate fractions.

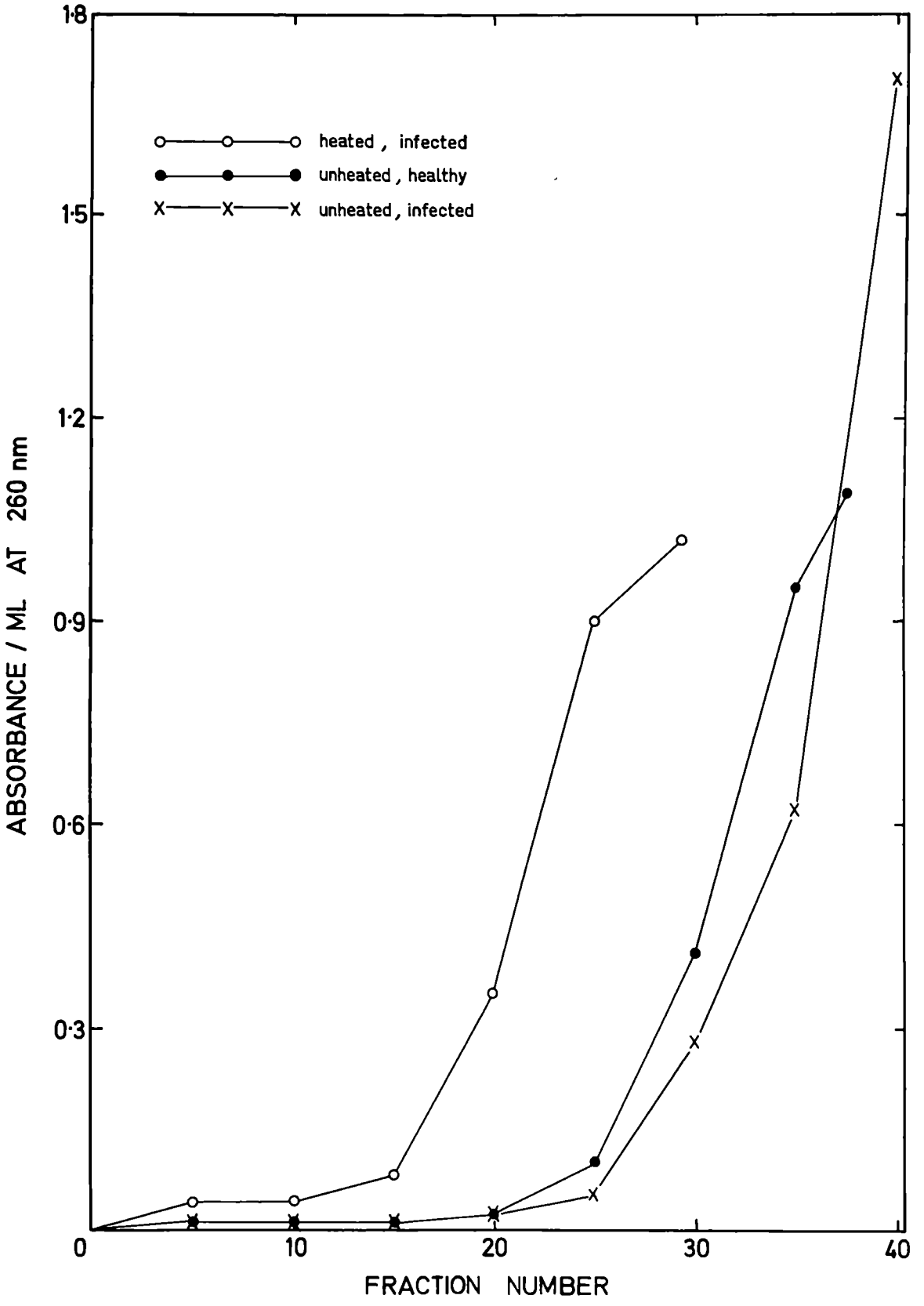

heated , infected
unheated , healthy
unheated , infected
ABSORBANCE / ML AT 260 nm
1·8
1·5
1·2
0·9
0·6
0·3
0
10
20
30
40
FRACTION NUMBER

TABLE 30.

Transformed adjusted mean lesion numbers induced on Chenopodium amaranticolor by homogenates from the roots and leaves of TAV infected tobacco plants grown in culture solutions in the presence and absence of actinomycin D for 14 days.

Treatment	T.A.M. Lesion No. / Half Leaf*	
	source of inoculum	
	leaves	roots
actinomycin D**	0.497	0.598
control	0.093	0.090
LSD (0.1%)	0.344	0.296

* T.A.M. = transformed adjusted mean; transformation was log (X+1); each figure was derived from the number of lesions induced on 12 half leaves.

** concentration of actinomycin D in the nutrient solutions was 1.0 µg / ml.

grown in the presence of the antibiotic, an observation which was independently reported by Hirai & Wildman (1967).

(b) TAV accumulation in excised leaves

The high cost of actinomycin D prevented further such studies on intact plants because of the large volumes of solutions required in nutrient culture. However, measurement of net virus accumulation in leaves or leaf discs floated on solutions, which would involve much smaller volumes, appeared feasible. Initially, therefore, net TAV accumulation was recorded in entire inoculated tobacco leaves floated either on:

1) half strength Knops solution
2) modified Long Ashton complete nutrient solution
3) 0.4 M sucrose
4) distilled water

These solutions were compared both alone and with the addition of 80 ppm benzimidazole, a synthetic cytokinin which delays the senescence of excised leaves.

Unfortunately, the homogenates derived from these leaves 14 days after inoculation induced no lesions when assayed for infectivity on C. amaranticolor, presumably due to their insensitivity at that time. However, intensity of virus symptoms was closely correlated with increases in fresh weight during the period of the experiment and the mean fresh weight of the leaves at harvest are therefore presented in Table 31 to give a probable indication of their virus content. Growth was greatest in the complete nutrient

TABLE 31.

Mean fresh weights (g) of TAV infected leaves after floating on various nutrient solutions for 14 days.

Nutrient Solution	Mean Fresh Weight of Leaves (g)*		Mean**
	benzimidazole present	benzimidazole absent	
modified Long Ashton	0.875	0.760	0.818
half strength Knops	0.490	0.530	0.510
distilled water	0.430	0.410	0.420
mean**	0.598	0.567	

* each figure represents the mean fresh weight of 3 leaves.

** difference between benzimidazole means not significant.
L.S.D. between nutrient solution means was:- 0.144 at 5%
0.203 at 1%
0.288 at 0.1%

solution and least in distilled water; this was correlated with their retention of colour by the time of harvest or, inversely, with their degree of senescence. The sucrose solutions proved unsatisfactory as a suspending medium because they enhanced microbial decay of the leaves. Benzimidazole increased the growth rate slightly, but the effect was not consistent and differences therefore were not significant.

(c) variable actinomycin D concentrations

Tobacco leaves were inoculated with TAV, excised, and floated on either modified Long Ashton solution or distilled water to which had been added either 0.25, 0.50, 0.75 or 1.00 µg / ml of actinomycin D. The concentration of TAV in the leaves was assayed on C. amaranticolor 14 days later.

The results, set out in Table 32, were inconsistent although observations on symptom intensity at the conclusion of the experiment paralleled those of apparent virus concentration, viz. symptoms on leaves floated on distilled water increased with actinomycin D concentration up to 0.75 µg / ml while no gradations were apparent among leaves floated on the complete nutrient solution containing variable levels of the antibiotic. The leaves floated on water were quite yellow at the time of harvest and it appeared that actinomycin D was more effective in stimulating viral synthesis in leaves under stress.

TABLE 32.

Transformed adjusted mean lesion numbers induced on Chenopodium amaranticolor by homogenates* from TAV infected tobacco leaves floated for 14 days on two nutrient solutions containing various concentrations of actinomycin D.

Concentration of Actinomycin D (µg / ml)	T.A.M. Lesion No. / Half Leaf**	
	nutrient solution***	
	distilled water	modified Long Ashton
0.25	0.705	1.248
0.50	0.925	1.457
0.75	1.297	1.030
1.00	0.665	1.227
LSD (5%)	0.330	0.225

* each homogenate was derived from 6 leaves.

** T.A.M. = transformed adjusted mean; transformation was log X; each figure was derived from the number of lesions induced on 12 half leaves.

*** difference between nutrient solution means was significant at 0.1%.

(d) TAV multiplication during heat treatment

Infective virus could never be detected in leaves on intact plants held at 36°C for 5 days when the treatment was commenced as soon as 24 hours after inoculation. However, infective virus was recovered from leaf discs, cut from plants growing in the glasshouse which had been inoculated 24 hours beforehand, incubated at 36°C for 5 days on 0.5% agar slopes in test tubes containing a high concentration (65 µg / ml) of actinomycin D. The infectivity of these discs was much lower than that of similar discs incubated at 25°C, but virus was consistently recovered from them whereas control discs kept at high temperature on agar without actinomycin D were always non-infective (Table 33).

Some indirect evidence that TAV also multiplies in leaf tissue in the absence of actinomycin D at 36°C was obtained by applications of cytokinins to plants during heat treatment. Batches of tobacco plants infected with TAV 14 days previously were placed in the phytotron cabinet and then sprayed daily with either 10^{-4} M kinetin, 10^{-4} M benzyladenine or water. After five days treatment, the relative infectivities of the plants were assayed on C. amaranticolor. The mean results over six experiments, set out in Table 34, show that the infectivity of kinetin-sprayed plants was much lower than that of the controls. Benzyladenine was without effect.

TABLE 33.

Retransformed adjusted mean lesion numbers induced on Chenopodium amaranticolor by homogenates from TAV infected leaf discs incubated at 25°C or 36°C for 5 days on agar slopes containing variable concentrations of actinomycin D.

Temperature	R.A.M. Lesion No. / Half Leaf*	
	concentration of actinomycin D (µg / ml)	
	0.0	65.0
25°C	34.4	12.6
36°C	0.0	2.6

* R.A.M. = retransformed adjusted mean; each figure was derived from the number of lesions induced on 12 half leaves; differences between the figures in each row and ~~in~~ column were significant at 0.1%.

TABLE 34.

Infectivities of leaf homogenates, as assayed on Chenopodium amaranticolor, from TAV infected tobacco plants heated at 36°C for 5 days when sprayed daily with either kinetin, benzyladenine or water.

Spray Treatment	R.A.M. Lesion No. / Half Leaf*	T.A.M. Lesion No. / Half Leaf**
kinetin	19.7	2.078
benzyladenine	47.9	2.170
water	42.6	2.154
	LSD (5%)	0.055
	LSD (1%)	0.077

* R.A.M. = retransformed adjusted mean.

** T.A.M. = transformed adjusted mean; transformation was log (X+100); each figure was derived from the number of lesions induced on 12 half leaves.

(e) rate of TAV synthesis at 36°C

Two attempts were made to measure the absolute rate of TAV synthesis in heated and unheated plants by measuring the incorporation of 5-^{3}H- uridine into its RNA in the presence of actinomycin D. Healthy plants were included as controls to check whether host RNA synthesis was completely suppressed.

The method used was based on that of Sanger & Knight (1963). Leaves from plants, some of which had been inoculated with TAV 5 days previously, were first dipped in vials containing 8.3 µg / ml of actinomycin D for 24 hours so that the final concentration within the plant would approximate 65 µg / g, this concentration inhibiting DNA transcription by more than 95%. The actinomycin D solution was then replaced with one containing 8 µc / ml of 5-^{3}H-uridine (22.8 c / mM) and incubation continued for a further 48 hours. Uridine tritiated in the no. 5 position of the pyrimidine ring was used so that there was no possibility of incorporation of label into DNA (Hayhoe & Quagline, 1965). Following incubation, individual leaves were frozen and ground in a chilled pestle and mortar. The RNA was then extracted by shaking the tissue vigorously for one minute in 45 ml 2:1 water saturated phenol: 0.1 M glycine buffer (pH 9.5 and containing 0.1 M sodium chloride, 0.005 M EDTA and 1% Wyoming bentonite). The phases were separated by centrifugation and 5 ml of the aqueous layer was poured into 15 ml of chilled ethanol. After standing overnight at

$0^{o}C$, the insoluble nucleic acid was collected on a Millipore filter, washed with ethanol, and then added to the scintillant.

The first experiment failed because of difficulties in the extraction procedure as grinding cold phenol, cold bentonite-buffer and chilled leaf tissue in a chilled pestle and mortar produced an unmanageable coacervate. This problem was overcome at the second attempt by grinding the chilled leaf tissue alone in a chilled pestle and mortar before quantitatively transferring the frozen powdered tissue to a centrifuge tube containing the buffer, bentonite and liquefied phenol.

The activities of RNA extracts obtained in this manner from heated and unheated, healthy and infected, plants are shown in Table 35. The results were inconclusive. They may indicate that either the uptake of actinomycin D was insufficient, or that its distribution within the plant was uneven so that the desired information could not be obtained.

XII KINETICS OF TAV INACTIVATION DURING HEAT TREATMENT

A knowledge of the kinetics of an inactivation may provide a clue as to how the inactivation occurs. Previous studies on the thermal inactivation of plant viruses, which have been reviewed, were done 'in vitro' at temperatures far above those used for heat treatment therapy, and there is no good reason to assume that the way in which inactivation proceeds under such conditions would be the same as that occurring in plants undergoing heat treatment.

TABLE 35.

Radioactivity of RNA extracted from tobacco leaves after incubation at 25° and at 36°C in 5-^{3}H- uridine in the presence of actinomycin D.

Temperature of Incubation	Infection	
	healthy	infected
25°C	770*	1031
36°C	824	577

* each figure is the mean cpm / g (fresh weight) from three plants; differences between infections and between temperatures are not significant.

Several attempts were made to establish the order of inactivation of TAV at 36°C both 'in vivo' and 'in vitro' by conventional methods. However, these all failed due to the difficulty in accurately measuring relative infectivities of different inocula, as was discussed earlier in section III (d).

I devised an alternative method for obtaining this information. The basis of the scheme ('dilution heating') is set out in Figure 15. For 'in vitro' studies, an infective sap sample was divided into two portions, and one of these was diluted 'n' times with buffer. These were then heated at 36°C for the same time after which the undiluted sample was diluted 'n' times. The infectivities of the two portions were then assayed on C. amaranticolor. The only result required from the assay is whether or not the two portions contain equal numbers of infective particles. If the portion heated in the more concentrated form is more, equally or less infective than the portion heated in the dilute form, then the inactivation is a zero, first or higher order reaction, respectively. Algebraic proofs for these statements are set out in Appendix 6.

Dilution heating of crude and chloroform-clarified leaf homogenates 'in vitro' at 36°C established that the inactivation was a second or higher order reaction (Tables 36, 37). These experiments were repeated three times, with similar results.

The system of dilution heating was also used for

FIGURE 15.

A flow-chart of the 'dilution heating' scheme devised for studying the kinetics of inactivation of TAV at 36°C.

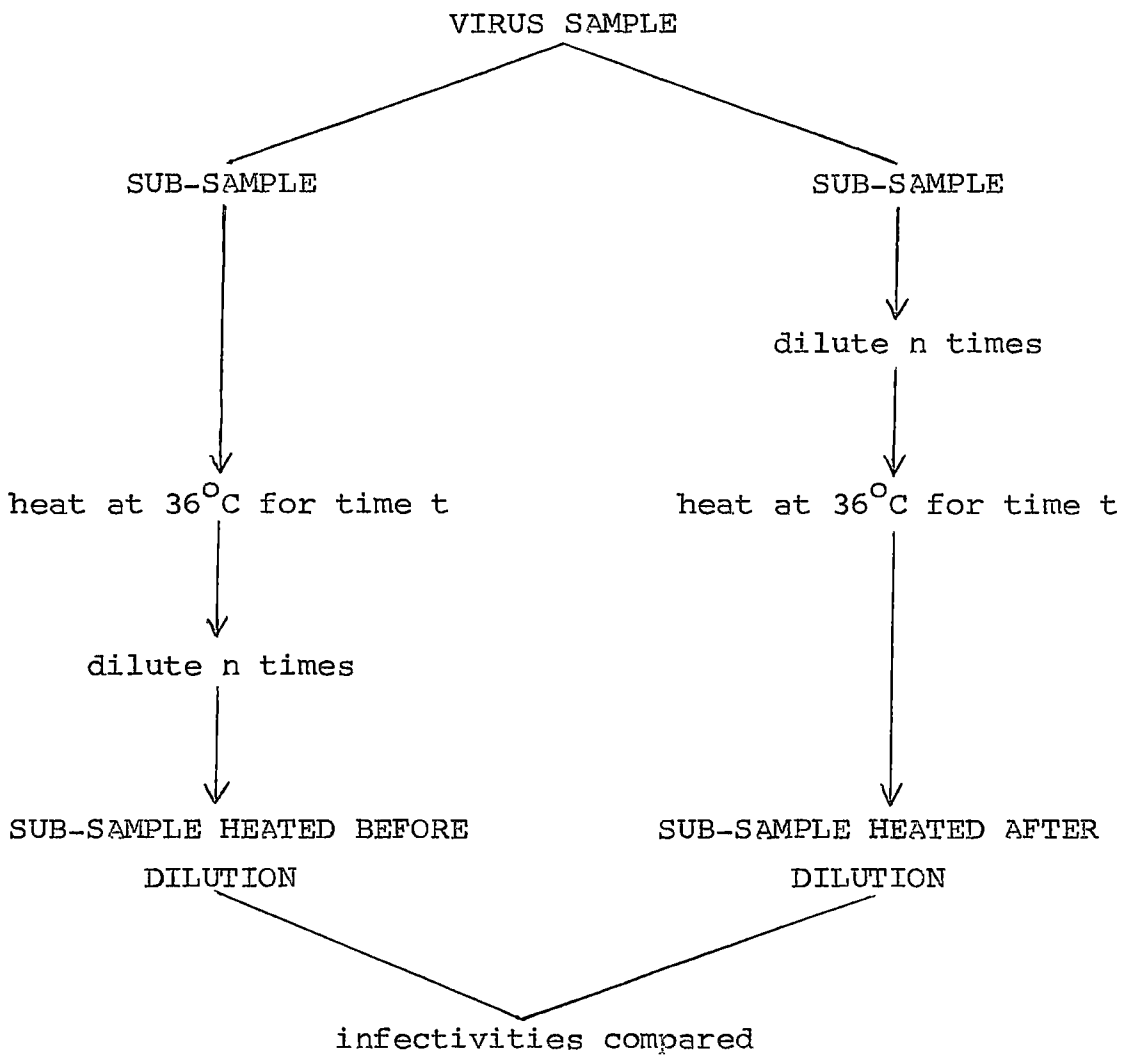

VIRUS SAMPLE
SUB-SAMPLE
SUB-SAMPLE
dilute n times
heat at 36°C for time t
heat at 36°C for time t
dilute n times
SUB-SAMPLE HEATED BEFORE DILUTION
SUB-SAMPLE HEATED AFTER DILUTION
infectivities compared

TABLE 36.

Infectivities of samples of a TAV-infected leaf homogenate diluted to varying extents with buffer, as assayed on Chenopodium amaranticolor, after heating at 36°C for 5 hours.

Concentration of Sap in Sample (%)	R.A.M. Lesion No. / Half Leaf*	T.A.M. Lesion No. / Half Leaf**
50.0	18.6	1.527
37.5	13.8	1.460
25.0	23.5	1.585
12.5	25.4	1.606
	LSD (5%)	0.079
	LSD (1%)	0.109

* R.A.M. = retransformed adjusted mean.

** T.A.M. = transformed adjusted mean; transformation was log (X+15); each figure was derived from the number of lesions induced on 12 half leaves; each sample was diluted with buffer, after heating, to give a sap concentration of 12.5% immediately prior to assay.

TABLE 37.

Infectivities of samples of a chloroform-clarified TAV infected leaf homogenate diluted to varying extents with buffer, as assayed on Chenopodium amaranticolor, after heating at 36°C for 5 hours.

Concentration of Sap in Sample (%)	R.A.M. Lesion No. /Half Leaf*	T.A.M. Lesion No. /Half Leaf**
50.0	6.8	0.831
37.5	7.0	0.846
25.0	8.1	0.907
12.5	10.6	1.026
	LSD (5%)	0.143

* R.A.M. = retransformed adjusted mean.

** T.A.M. = transformed adjusted mean; transformation was log (X+1); each figure was derived from the number of lesions induced on 12 half leaves; each sample was diluted with buffer, after heating, to give a sap concentration of 12.5% immediately prior to assay.

'in vivo' studies. Thirty tobacco plants growing in the glasshouse, which had been inoculated 14 days previously, were split into three groups of 10. The first group (x) were triturated in buffer, clarified with chloroform, and stored on ice for two days when the infectivities of dilutions of this extract were compared with a clarified extract prepared from the second group of plants (y) after they had been heat treated 36°C for this period. The third group of plants (z) were heated for a further two days after which the infectivity of the clarified extract obtained from them was compared with dilutions of the extract from group y which had been held on ice for the two days.

The results, set out in Table 38, were not completely clear-cut because some virus inactivation occurred while the clarified extracts were stored on ice. However, the fact that the infectivity ratio of x:y was so much greater than that of y:z indicates that inactivation during heat treatment is almost certainly higher than a first order reaction.

XIII RATES OF INACTIVATION IN DIFFERENT SYSTEMS

Results were available from 8 separate experiments conducted during this study which enabled comparisons to be made between the relative rates of inactivation 'in vivo' and 'in vitro'. Individual homogenates were derived from at least 8 plants in every instance.

The rate of TAV inactivation was generally much faster 'in vitro', but the differential between the two

TABLE 38.

The relative infectivities of chloroform-clarified homogenates from TAV infected tobacco plants in an 'in vivo' dilution heating experiment, as assayed on Chenopodium amaranticolor.

Test No.	Inoculum*	R.A.M. Lesion No. / Half Leaf**	Relative Infectivities***
1	x	326.5	x : y = 1 : 1.20
	y	185.4	
2	y'	5.0	y' : z = 1 : 0.63
	z	24.9	

* x = homogenate from an unheated group of plants stored on ice for 2 days.
y = homogenate from a group of plants heated at 36°C for 2 days.
y' = homogenate from a group of plants heated at 36°C and stored on ice for 2 days.
z = homogenate from a group of plants heated at 36°C for 4 days.

** R.A.M. = retransformed adjusted mean; each figure was derived from the number of lesions induced on 12 half leaves.

*** obtained by comparing serial dilutions of x with y, and serial dilutions of y' with z.

systems varied greatly between experiments (Table 39). Other 'in vitro' tests showed that the rate of inactivation in crude leaf extracts was normally much greater than that in aliquots clarified with chloroform prior to heating (Table 40).

There was only a single opportunity to examine the inactivation of TAV in purified preparations. This showed that the infectivity of an aliquot heated at 36°C for 1 hour was just slightly greater than that of an unheated sample (Table 41). Examination of these two aliquots in the analytical ultracentrifuge did not provide any evidence for the release of RNA from within intact TAV particles during heating (Plate 7).

XIV THE ROLE OF PHENOLICS IN INACTIVATION

(a) polyphenoloxidase assay method

The method used was adapted from that used by Sisler & Evans (1958). The enzyme extraction was performed at 0°C and the assay at 20°C. All lamina tissue from each plant was weighed and triturated in 0.1 M potassium phosphate (pH 7.0). The homogenate was quantitatively transferred with additional buffer to a centrifuge tube. After centrifugation, the clarified supernatant was collected and chilled acetone added to 37% by final volume. The precipitate was collected by centrifugation and the soluble portion resuspended in 0.1 M potassium phosphate (pH 7.0) to serve as the enzyme extract. The assay mixture, in a final volume of 3 ml, consisted of 0.1 M potassium phosphate

TABLE 39.

Comparisons between the loss of TAV infectivity when heated at 36°C 'in vivo' and 'in vitro' for varying periods of time.

Test No.	Period of Treatment (hours)	R.A.M. Lesion No. / Half Leaf*	
		heated 'in vitro'	heated 'in vivo'
1	1	88.2	98.8
2	1	41.1	51.1
3	2	127.1	109.7
4	2	47.6	57.1
5	3	120.3	140.0
6	3	0.3	59.5
7	4	119.6	130.7
8	4	55.7	160.2

* R.A.M. = retransformed adjusted mean; in each test the figures were derived from the number of lesions induced on not less than 12 half leaves of Chenopodium amaranticolor.

TABLE 40.

Comparisons between the loss of TAV infectivity when heated at 36°C 'in vitro' for varying periods of time before and after clarification with chloroform.

Test No.	Period of Treatment (hours)	R.A.M. Lesion No. / Half Leaf*	
		chloroform clarification	
		after heating	before heating
1	1	81.1	78.4
2	2	94.0	109.9
3	3	122.4	120.3
4	4	73.9	119.6
5	5	97.4	136.9

* R.A.M. = retransformed adjusted mean; in each test the figures were derived from the number of lesions induced on not less than 12 half leaves of Chenopodium amaranticolor.

TABLE 41.

The infectivity of a purified TAV preparation after heating at 36°C for 1 hour compared with that of an unheated aliquot.

Treatment	R.A.M. Lesion No. / Half Leaf*
heated	374.3
unheated	308.5

* R.A.M. = retransformed adjusted mean; each figure was derived from the number of lesions induced on 24 half leaves of Chenopodium amaranticolor, the difference between means was significant at 5%.

(pH 7.0), 5.7×10^{-5} M chlorogenic acid, 10^{-3} M EDTA, 2.8×10^{-5} M ascorbic acid (freshly prepared in EDTA) and enzyme extract. Sufficient enzyme extract was added to give a decrease of about 0.07 absorbance units per minute, when measured at 265 nm.

Polyphenoloxidase (PPO) concentration was expressed as decrease in absorbance at 265 nm between 30 and 90 seconds after the reaction was commenced on the basis of 1 ml of enzyme extract in the reaction mixture which was derived from 1 g of fresh leaf tissue whose acetone precipitate had been resuspended in 10 ml of buffer. Correction was made for any small amount of ascorbic acid oxidase in the extract by omitting chlorogenic acid from the reaction mixture.

(b) chlorogenic acid assay method

Chlorogenic acid, (CA), which is the major phenolic constituent of tobacco leaves (Shiroya et al., 1955), was determined by a method adapted from that of Zucher & Ahrens (1958) which is based on the fact that a bright red pigment is formed when alkali is added to the reaction product of nitrous and chlorogenic acids.

All lamina tissue from each plant was weighed and triturated in chilled acetone. The homogenate was clarified by centrifugation and the supernatant made up to 50 ml with water. An aliquot of this extract, containing between 0.1 and 1.0 μ moles of chlorogenic acid, was then placed on a column of chromatographic grade alumina (3 g in a 50 ml burette). The column was washed successively with 5 ml

water, 8 ml nitrous acid (freshly made by mixing 4 ml 0.5% sodium nitrite and 4 ml 5% acetic acid), and 5 ml water. Ten ml 5 N sodium hydroxide was then run through the column together with additional water washings. The red coloured eluate was collected, made up to 50 ml with water, and the absorbance measured at 525 nm. A standard curve was constructed by subjecting aqueous chlorogenic acid solutions to the above procedure.

(c) PPO and CA changes at 36°C

Extracts from heat-treated plants are much darker than those from their respective controls (Plate 16), suggesting higher concentrations of PPO, or CA, or both. Therefore, experiments were designed to follow changes in the level of this enzyme and its substrate in heated plants, as the infectivity of TAV in leaf extracts is increased if reducing agents are included in the homogenising buffer (Table 7). At the same time, the possibility was investigated that the decline in specific infectivity of TAV some time following inoculation might be due to changes in this system.

Half of a batch of 90 uniform young tobacco plants were inoculated with TAV. Three plants were individually assayed for PPO at the time of inoculation and further groups of three healthy and three infected plants were assayed at 3-day intervals up to 15 days. At this time, half of the remaining plants were placed in the phytotron cabinet and samples of three plants from the four infection-

PLATE 16.

A chloroform clarified preparation extracted from tobacco plants after heat treatment at 36°C for 5 days compared with an extract from unheated plants on the left hand side.

environment combinations were assayed at 3-day intervals for a further 15 days.

A second batch of 93 plants were treated in a similar way and analysed for CA content.

The results of the PPO assays are shown in Figures 16 and 17. They are presented in the two ways, i.e. on a linear and a logarithmic scale, so that the increase in enzyme content following infection is apparent and so that the dramatic increase in PPO in heat-treated plants is realistically displayed. Statistical analysis of the results is set out in Appendix 7.

The results of the CA analyses are depicted in Figure 18. The CA content was inversely correlated with level of PPO, although the differences were less dramatic. This strongly suggests that CA was being oxidised 'in vivo' during heat treatment. Statistical analysis of the results is set out in Appendix 8.

(d) effect of temperature on PPO activity

Enzyme reaction rates generally increase with temperature until the temperature is approached at which the enzyme is denatured. However, the action of PPO requires the presence of oxygen and the solubility of this gas in water decreases with increasing temperature. The rate of oxidation of CA by PPO was therefore measured over a wide temperature range using one of the extracts from the previous experiment. The results (Figure 19) show that the reaction proceeds fastest at 20^{o}C. However, the rate at temperatures

FIGURE 16.

The relative polyphenoloxidase content* of lamina tissue from tobacco plants at varying times after inoculation and after varying periods of heat treatment.

* each point on the graph represents the mean value determined for 3 healthy and 3 infected plants; the units on the ordinate are as defined in the last paragraph of section XIV(a).

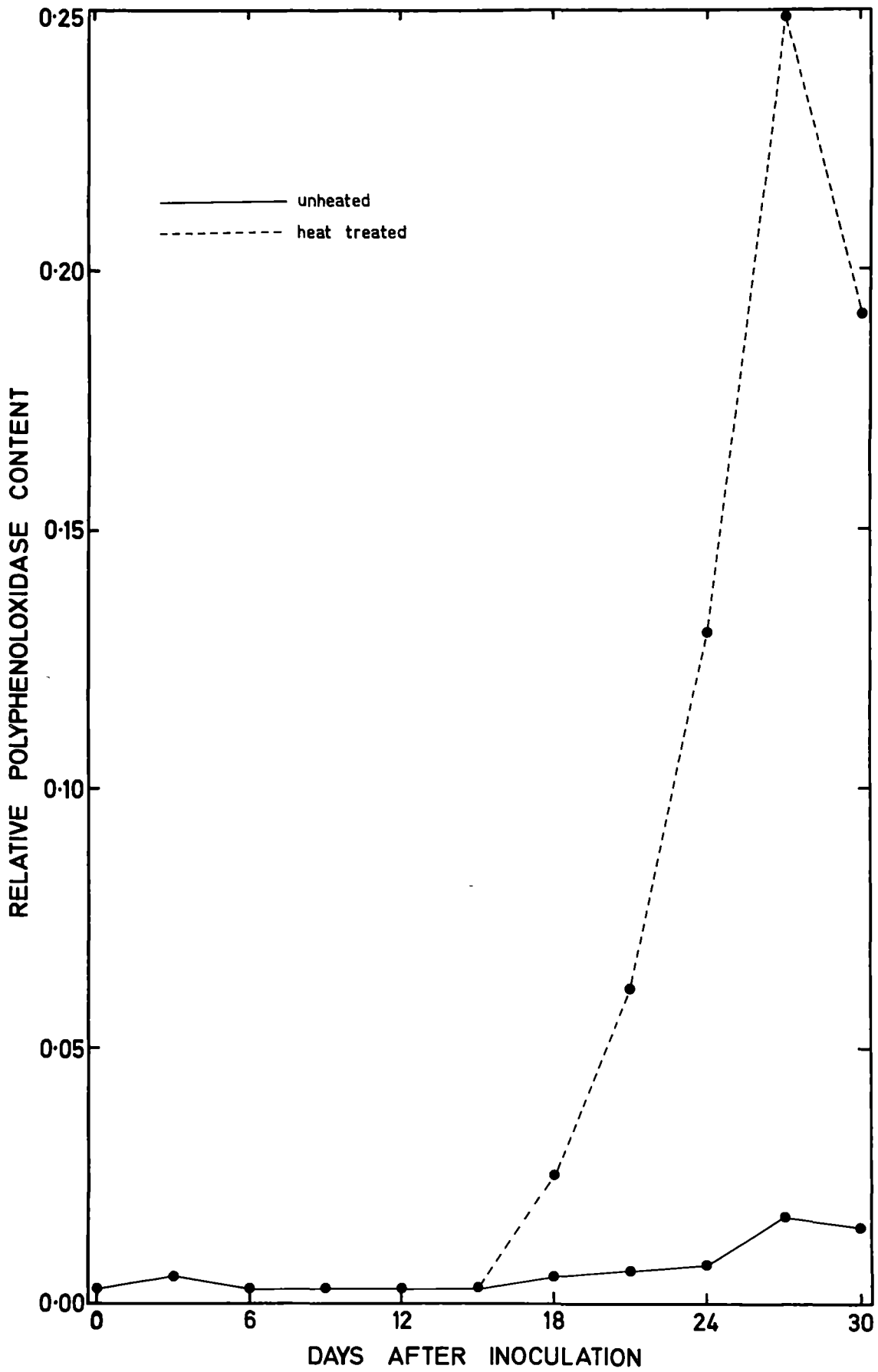

0·25
0·20
0·15
0·10
0·05
0·00
RELATIVE POLYPHENOLOXIDASE CONTENT
unheated
heat treated
0
6
12
18
24
30
DAYS AFTER INOCULATION

FIGURE 17.

The relationship, depicted on a logarithmic scale, between the polyphenoloxidase content* of lamina tissue from tobacco plants at varying times after inoculation and after varying periods of heat treatment compared with that of tissue from uninoculated plants.

* each point on the graph represents the mean value determined for 3 plants; the units on the ordinate are $[3 + \log (x + 0.001)]$ where x is the unit of polyphenoloxidase concentration as defined in the last paragraph of section XIV(a).

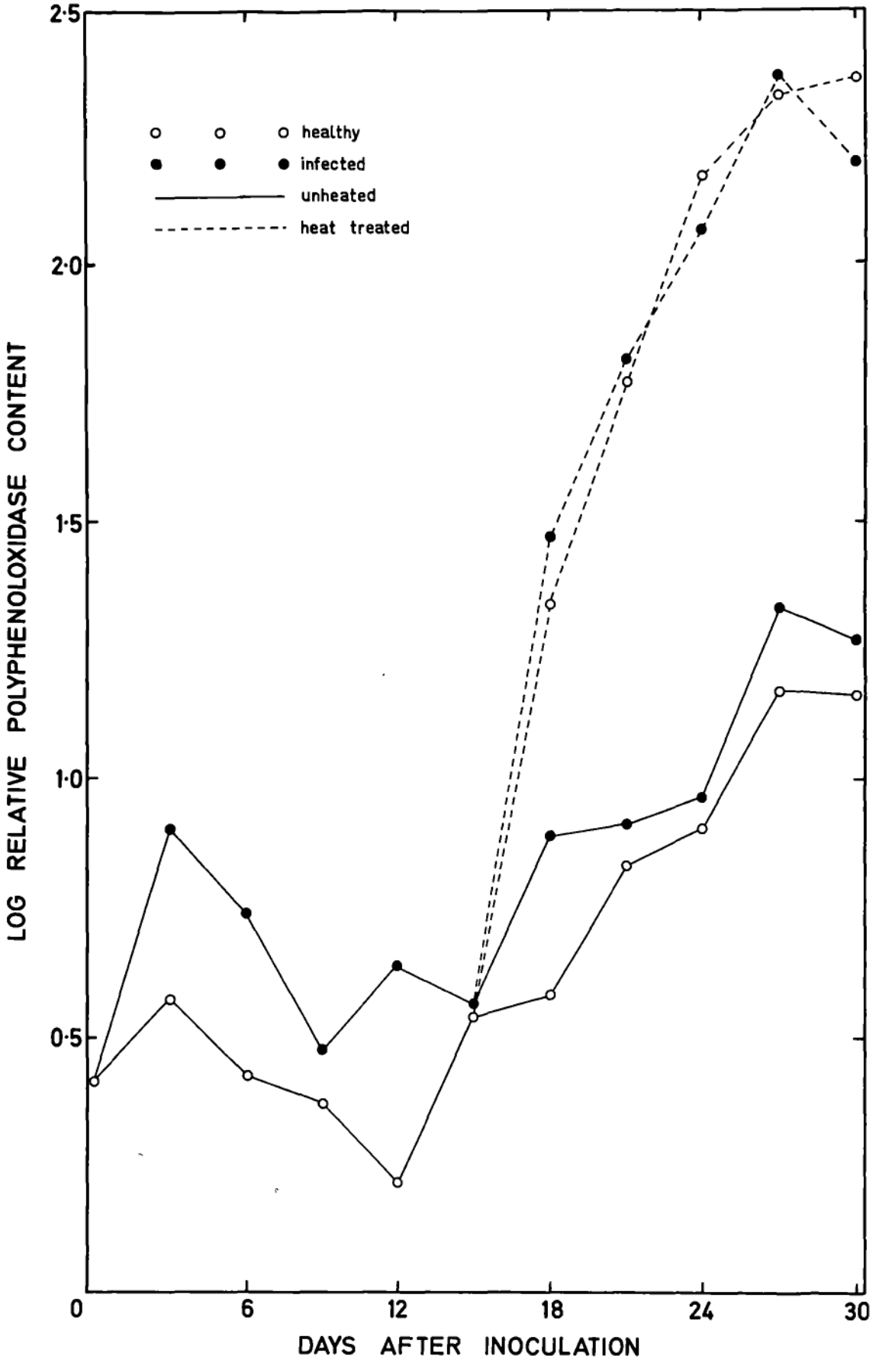

2·5
2·0
1·5
1·0
0·5
0
6
12
18
24
30
healthy
infected
unheated
heat treated
LOG RELATIVE POLYPHENOLOXIDASE CONTENT
DAYS AFTER INOCULATION

FIGURE 18.

The chlorogenic acid content of lamina tissue from tobacco plants at varying times after inoculation and after varying periods of heat treatment compared with that of tissue from uninoculated plants.

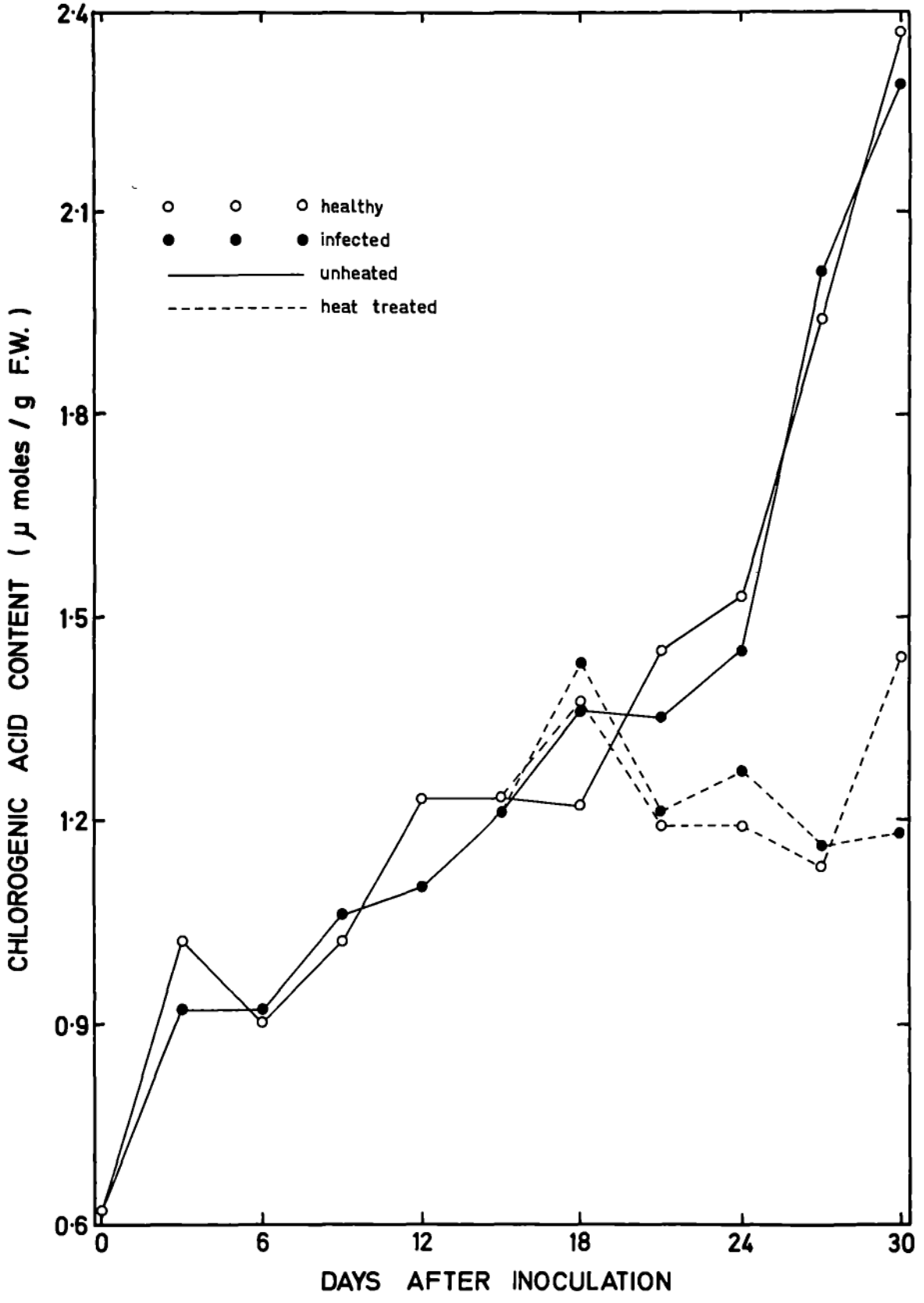
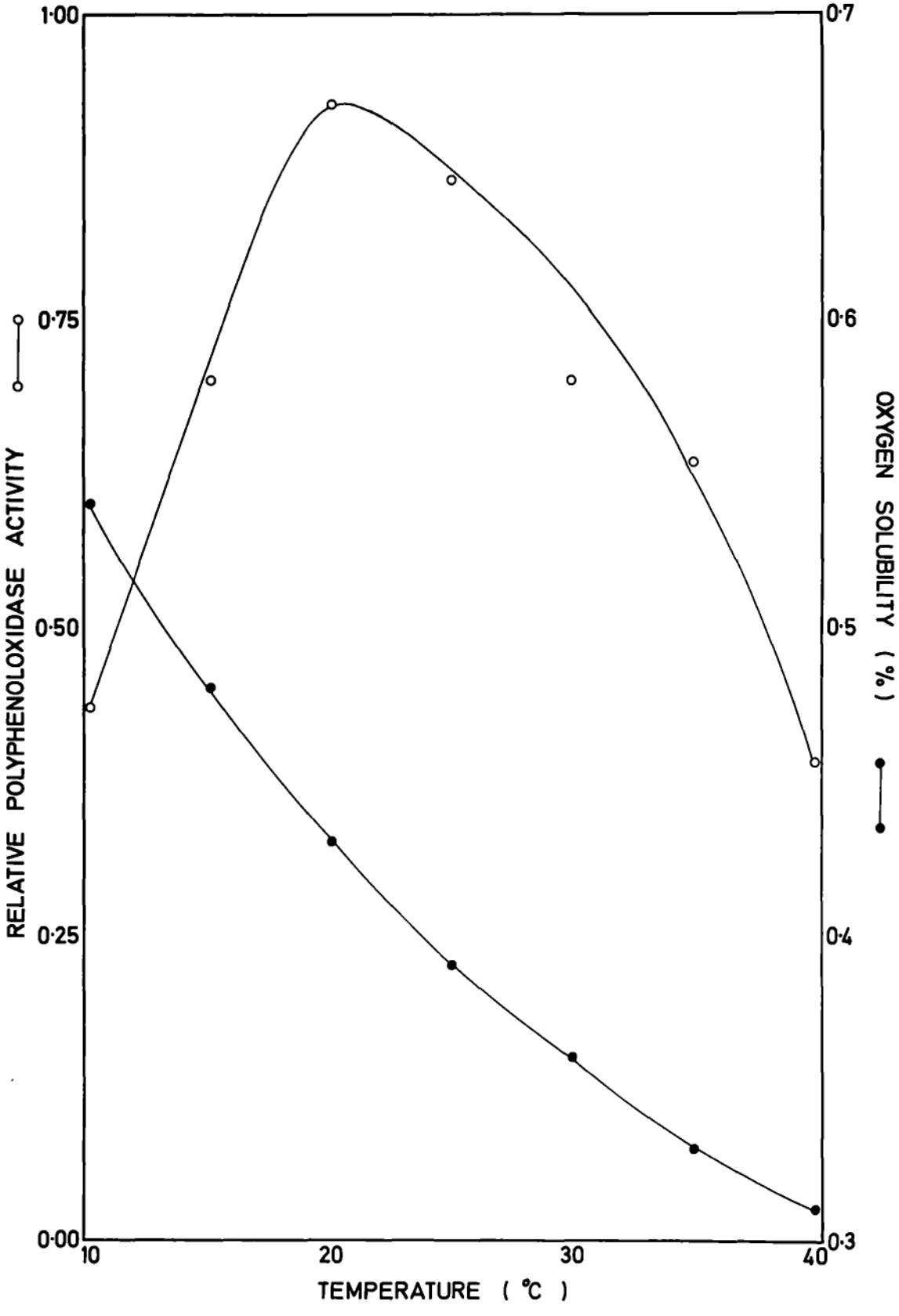

2·4
2·1
1·8
1·5
1·2
0·9
0·6
0
6
12
18
24
30
healthy
infected
unheated
heat treated
CHLOROGENIC ACID CONTENT (μ moles / g F.W.)
DAYS AFTER INOCULATION

FIGURE 19.

The effect of temperature on the rate of oxidation of chlorogenic acid by tobacco leaf polyphenoloxidases and on the solubility of oxygen in water*.

* data for the solubility of oxygen in water was obtained from Hodgman (1944).


1·00
0·75
0·50
0·25
0·00
RELATIVE POLYPHENOLOXIDASE ACTIVITY
0·7
0·6
0·5
0·4
0·3
OXYGEN SOLUBILITY (%)
10
20
30
40
TEMPERATURE (°C)

near 36°C was still significant.

(e) application of PPO and CA to infected plants

Groups of uniform tobacco plants in the glasshouse, which had been inoculated 14 days previously, were sprayed daily for five days with either 10^{-3} M cysteine, 20 µg / ml mushroom tyrosinase (Koch-Light), 10^{-3} M chlorogenic acid, or water. TAV was then extracted from samples of the lamina tissue from each plant, to assay for infectivity on C. amaranticolor, in both of two different ways in order to minimise 'in vitro' changes - by the normal method in the cold with cysteine containing buffer, and by including alumina (1:10, W:W) in the mortar to adsorb all CA. It had been established previously that this proportion of alumina did not adsorb any detectable quantity of TAV. This experiment, replicated in each instance, was repeated six times.

The extracts produced in the two ways had similar infectivities. The mean results (Table 42) show that both CA and mushroom tyrosinase markedly reduced infectivity. Cysteine also reduced infectivity, although not to the same extent as the other substances, probably due to oxidation of the solution to cystine during storage over the period of the experiment. It was later established that cysteine is readily autoxidised in air at normal temperature.

(f) application of chlororesorcinol to plants at 36°C

Twenty young tobacco plants growing in the glasshouse, which had been inoculated with TAV 14 days

TABLE 42.

Infectivities of leaf homogenates, as assayed on Chenopodium amaranticolor, from TAV-infected tobacco plants after daily spraying for 5 days with either water, mushroom tyrosinase, chlorogenic acid or cysteine hydrochloride.

Treatment	Adjusted Mean Lesion No. / Half Leaf
water	251
tyrosinase	191
chlorogenic acid	204
cysteine	214
LSD (5%)	33
LSD (1%)	44

previously, were placed in the phytotron cabinet. Half of them were sprayed daily with 10^{-3} M chlororesorcinol, an 'in vivo' inhibitor of PPO (Kull et al., 1954), for 5 days and their infectivity was then compared with that of plants sprayed only with water. The results indicated that the effect of heat treatment in reducing the level of infective TAV is partially reversed if the activity of PPO is reduced (Table 43).

XV THE ROLE OF RIBONUCLEASES IN INACTIVATION

(a) ribonuclease assay method

The method used was developed from those used by Bagi & Farkas (1967) and Frisch-Niggenmeyer & Reddi (1967). Frozen leaf tissue was ground in a chilled pestle and mortar, quantitatively removed to a centrifuge tube, thawed, and clarified by centrifugation at 0°C. The reaction mixture consisted of 0.2 ml of the clarified supernatant, 0.5 ml 1% RNA (from yeast, high polymerised; Calbiochem) in 0.1 M acetate buffer (pH 5.1) and 0.3 ml acetate buffer. The mixture was incubated at 37°C for 1 hour after which the reaction was stopped by adding 0.37 ml of cold 2 N hydrochloric acid. Acid added to tubes of the reaction mixture prior to incubation served as controls. In addition, controls without RNA were included to allow for increases in absorption due to polyphenoloxidase action. The tubes were held overnight at 0°C following addition of the hydrochloric acid. Insoluble nucleic acid was then removed by centrifugation, and an aliquot of the supernatant

TABLE 43.

Infectivities of leaf homogenates, as assayed on Chenopodium amaranticolor, from TAV-infected tobacco plants grown at 36°C for 5 days after daily spraying with either 10^{-3} M chlororesorcinol or with water.

Treatment	Mean Lesion No. / Half Leaf*	S.E.**
chlororesorcinol	204.30	18.27
control	147.95	9.21

* each figure was derived from the number of lesions induced on 20 half leaves of Chenopodium amaranticolor.

** S.E. = standard error.

diluted ten-fold prior to reading the absorbance at 260 nm. An optical density of 0.01 was taken as one enzyme unit.

(b) effect of RNase on TAV

Aliquots of an homogenate from TAV infected plants were incubated at room temperature with RNase (bovine pancreas, 5 x crystallised; from Calbiochem) at final concentrations of either 0, 0.001, 0.01 or 0.1 µg / ml.

Infectivity was reduced by incubation with RNase (Table 44). Incubation of homogenates for varying periods of time established that the effect of the enzyme was on the virus rather than on a competitive effect between the enzyme and virus for infectible sites on the assay host. Further support for this contention came from the findings that inocula were slightly more infective when prepared in the presence of RNA (Table 45). This latter result provided evidence that the concentration of RNase in Hickory Prior homogenates is sufficiently high to affect TAV infectivity. However, inocula prepared in the presence of phenol or diethylcarbonate were less infective than those prepared with buffer alone (Table 46).

(c) changes in concentration of RNase

An experiment, whose design was identical to that described in section XIV(c), was done to measure the concentration of RNase in tobacco plants at various times after inoculation and commencement of heat treatment.

No results were obtained because the samples were lost due to a failure of the deep freeze unit in which they

TABLE 44.

Infectivities of aliquots of an homogenate from TAV infected tobacco plants, as assayed on Chenopodium amaranticolor, when incubated for 1 hour at room temperature with varying concentrations of ribonuclease.

RNase (µg / ml)	R.A.M. Lesion No. / Half Leaf*	T.A.M. Lesion No. / Half Leaf**
0	13.2	1.151
0.001	14.7	1.197
0.01	2.5	0.544
0.1	2.6	0.551
	LSD (5%)	0.200
	LSD (1%)	0.274
	LSD (0.1%)	0.377

* R.A.M. = retransformed adjusted mean.

** T.A.M. = transformed adjusted mean; transformation was log (X+1); each figure was derived from the number of lesions induced on 12 half leaves of Chenopodium amaranticolor.

TABLE 45.

Infectivities of leaf homogenates, as assayed on Chenopodium amaranticolor, extracted from TAV infected tobacco plants with buffer containing either RNA, RNase or neither.

Treatment	Mean Lesion No. / Half Leaf*	S.E.
buffer control (BC)	119.7	16.1
BC + 4mgm / ml RNA	129.8	20.0
BC + 20 µg / ml RNase	0.0	0.0

* each figure was derived from the number of lesions induced on 16 half leaves of Chenopodium amaranticolor.

TABLE 46.

(a) Comparison between the infectivities of TAV inocula extracted from tobacco leaves in the presence of buffer and a phenol-buffer mixture.

Treatment	Mean Lesion No. / Half Leaf
buffer extract	49.9*
phenol-buffer extract	3.1

* difference significant at 1%.

(b) Comparison between the infectivities of TAV inocula extracted from tobacco leaves in the presence of buffer and a diethylcarbonate-buffer mixture.

Treatment	Mean Lesion No. / Half Leaf
buffer extract (BE)	17.1*
BE + diethylcarbonate	13.6

* difference significant at 5%.

were being stored pending assay. Therefore, the experiment was repeated on a much smaller scale, and the results, set out in Table 47, indicate that the RNase concentration increases markedly in Hickory Prior plants during heat treatment.

XVI PPO, CA, RNase AND TAV CONCENTRATION IN VARIOUS TOBACCO HOSTS

The aim of this experiment was to examine whether the rate of TAV accumulation at normal temperature and inactivation at high temperature differed among a range of tobacco hosts. The thought was that if differences could be shown to exist, then correlations between these and the normal and any heat induced increased levels of PPO, CA and RNase might provide a clue as to the relative importance of these factors in TAV inactivation during heat treatment. The types of tobacco used were Nicotiana clevelandii, N. glutinosa, and N. tabacum (varieties Atropurpureum, Hickory Prior, Turkish, White Burley, and Xanthi).

Batches of these plants were raised at the same time and infected together with the same inoculum. Sufficient plants were used so that a bulked sample of all leaves from five plants could be used for each determination. The plants were kept on the glasshouse bench for 14 days after inoculation when half of them were harvested to assay for infectivity, PPO, CA and RNase. At this time, the severity of symptoms on the plants were, in decreasing

TABLE 47.

Concentration of ribonuclease (units / g D.W.) in extracts from healthy tobacco leaf tissue heated at 36°C for varying periods of time compared with that in extracts from unheated tissues.

Time of Treatment (days)	Mean Concentration of Ribonuclease (units / g D.W.)*	
	heated	unheated
0	587	587
3	881	858
6	1443	1306
9	2003	1284

* definition of a unit of RNase concentration is set out in section XV(a); each figure within the table was derived from estimations on two individual plants.

order:- N. clevelandii, White Burley, N. glutinosa, Hickory Prior, Xanthi, Atropurpureum, and Turkish. These observations were correlated with infectivity as determined by inoculation to Vigna sinensis var. Blackeye (Table 48).

The remaining plants were then held at 36°C for a further 5 days before harvesting. None of the inocula prepared at this time induced lesions on the insensitive 'Blackeye' cowpea which had to be used due to the unavailability of C. amaranticolor at that time. During the infectivity assay, it was noted that inocula prepared from N. clevelandii, N. glutinosa and White Burley showed little sign of oxidation whereas those prepared from the four other tobacco varieties were moderately dark.

All leaf samples taken both before and after heat treatment for PPO, CA and RNase assay were lost due to an unsuspected failure of the deep freeze unit in which they were being stored until time allowed for their determination, apart from six samples for CA taken after heat treatment. These results, set out in Table 49, indicate that large differences occurred in the CA content among the varieties tested.

XVII pH AND IONIC STRENGTH OF EXTRACTS FROM PLANTS DURING HEAT TREATMENT

The changes in the dry weight : fresh weight of heat-treated plants, described in sections VI and VII, prompted investigations of the pH and ionic strength of sap

TABLE 48.

The infectivities of leaf homogenates, as assayed on Vigna sinensis, extracted from seven tobacco hosts 14 days after they had been inoculated with TAV.

Host	R.A.M. Lesion No. /Half Leaf**	T.A.M. Lesion No. /Half Leaf***
White Burley	92.30	1.9699
N. clevelandii	70.01	1.8513
Hickory Prior	1.99	0.4763
N. glutinosa	1.30	0.3620
Xanthi	1.25	0.3517
Atropurpureum	0.99	0.2983
Turkish	0.51	0.1800
	LSD (5%)	0.1506
	LSD (1%)	0.2015
	LSD (0.1%)	0.2645

** R.A.M. = retransformed adjusted mean.

*** T.A.M. = transformed adjusted mean; the transformation was log (X+1); each figure was derived from the number of lesions induced on 24 leaf halves.

TABLE 49.

Chlorogenic acid content of the lamina tissue from six tobacco varieties after treatment at 36°C for 5 days.

Variety	Chlorogenic Acid (μ moles / g F.W.)
Nicotiana glutinosa	0.85
White Burley	1.43
Turkish	1.52
Atropurpureum	1.95
Xanthi	2.00
Hickory Prior	2.41

extracted from plants during heat treatment, as changes in these factors are known to alter the stability of some spherical viruses (see review).

(a) pH of infected plants

Half of a group of 80 tobacco plants, all of which had been inoculated with TAV 14 days previously, were placed in the phytotron cabinet. Groups of five plants from both the glasshouse and cabinet were harvested daily for 8 consecutive days and triturated in an equal volume of water. The pH of the extract was measured without further treatment. The pH of sap from the heated plants fell markedly with time of treatment (Figure 20; statistical analysis in Appendix 9).

(b) pH of healthy plants

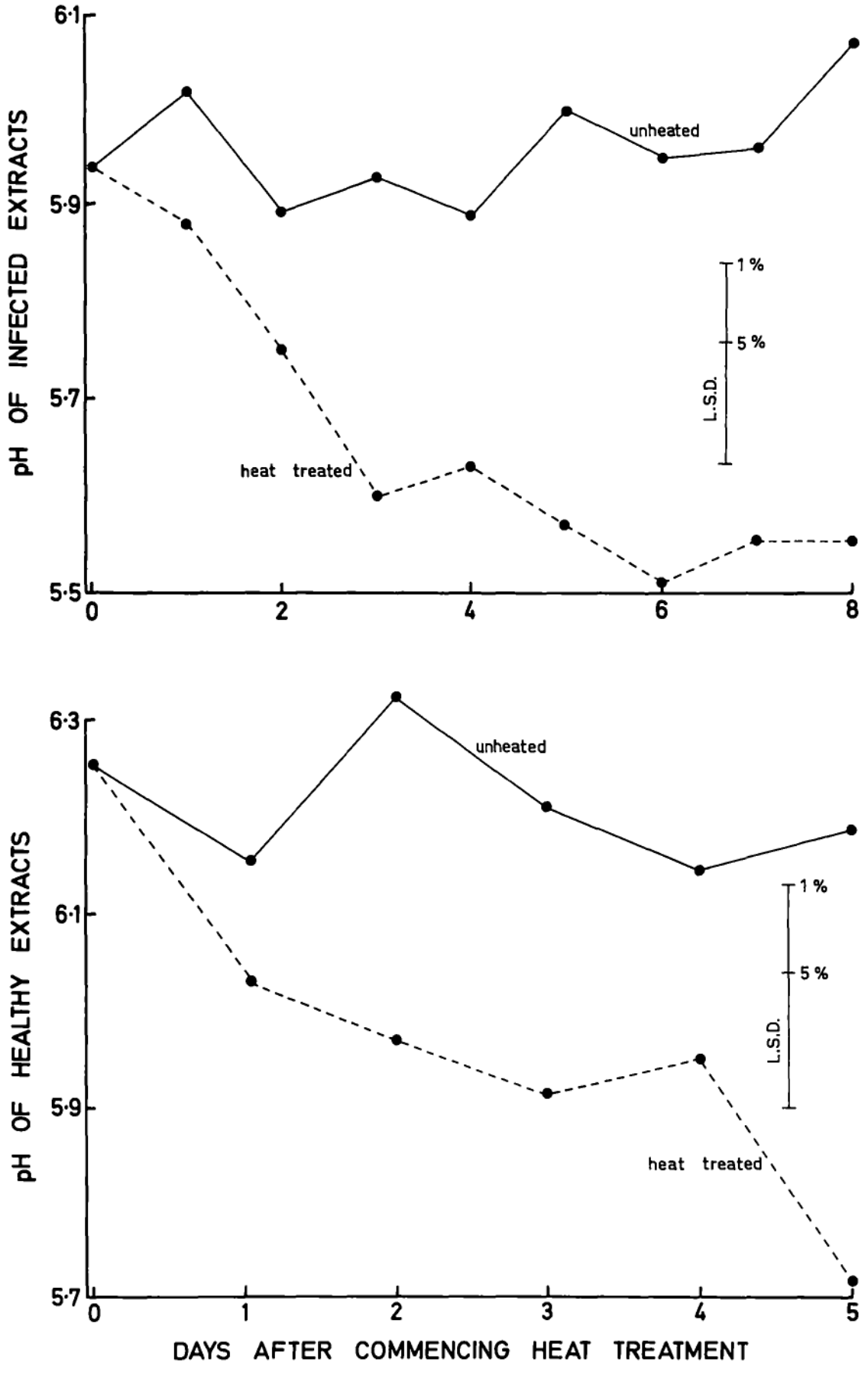
The design of the experiment was identical to that for the infected group except that there were only sufficient plants to run the experiment for five days. The pH again fell dramatically with time of treatment (Figure 20; statistical analysis in Appendix 9).

(c) ionic strength of extracts from plants during heat treatment

A preliminary experiment to measure the ionic strength of plant sap extracted from plants grown in the usual way produced extremely variable results, presumably due to differences in the water status of the container-grown plants between times of sampling. However, there were indications that the electrical resistance of sap from heated plants was much higher than that from the

FIGURE 20.

The pH of sap extracts from healthy and TAV-infected tobacco plants after varying periods of heat treatment compared with that of extracts from unheated plants.


pH OF INFECTED EXTRACTS
6·1
5·9
5·7
5·5
0
2
4
6
8
unheated
heat treated
1 %
5 %
L.S.D.
pH OF HEALTHY EXTRACTS
6·3
6·1
5·9
5·7
0
1
2
3
4
5
unheated
heat treated
1 %
5 %
L.S.D.
DAYS AFTER COMMENCING HEAT TREATMENT

controls.

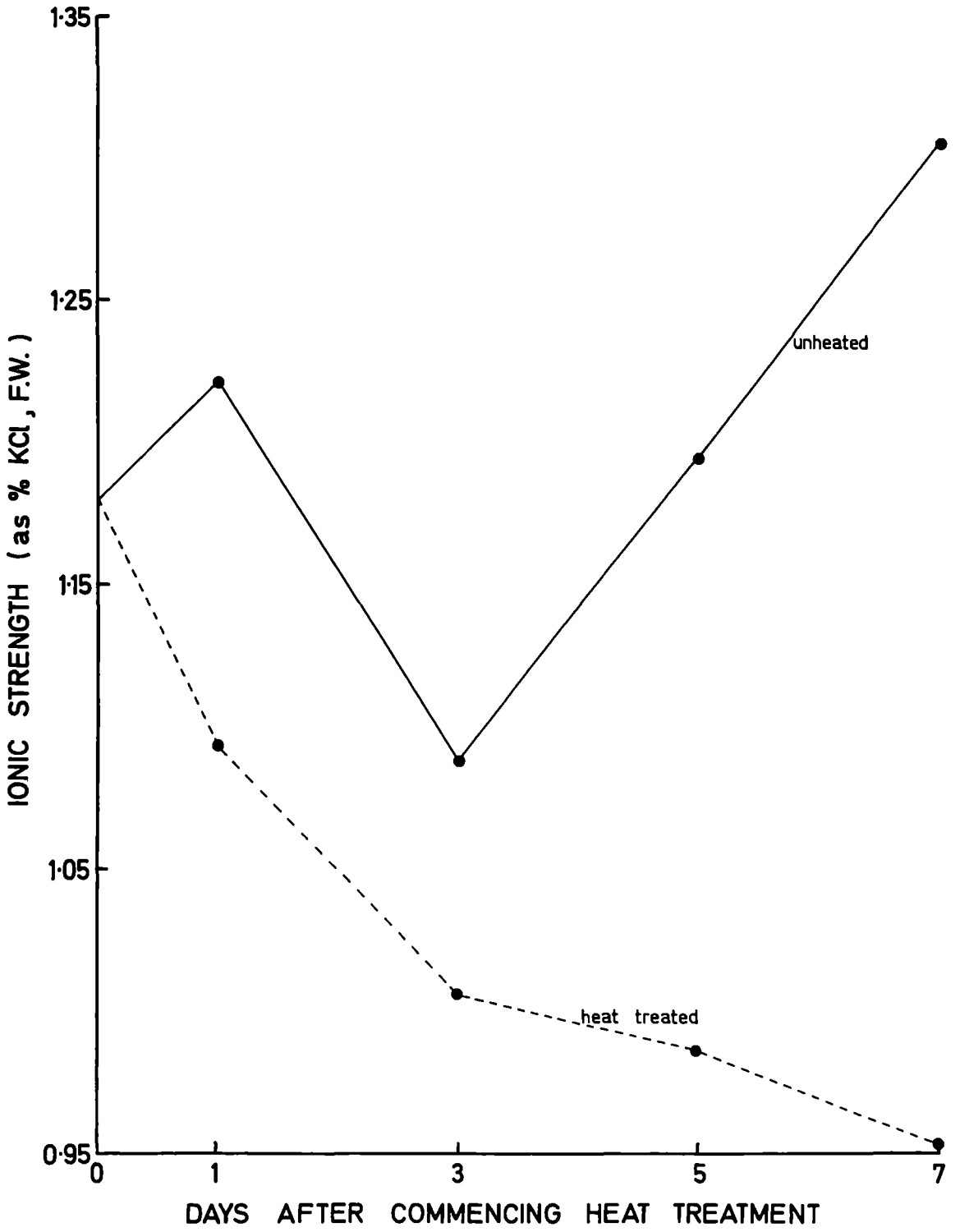
Therefore, an experiment using 54 plants growing in solution culture was designed in an attempt to overcome this problem. Half of them were inoculated with TAV, and 14 days later, 12 infected and 12 of the healthy plants were placed in the phytotron cabinet. Three plants from each temperature-infection combination were harvested after 1, 3, 5 and 7 days for analysis. This was done by triturating the lamina tissue from each plant in distilled, deionised water and making up the homogenate to a final dilution of 1:5000, W:V. The resistance of these suspensions was then measured with a 'home-made' meter, essentially a Wheatstone bridge circuit, one arm of which was a Mullard conductivity cell. Adjustment was made to the measured conductivities for the temperature of the suspension, using $20^{o}C$ as the standard temperature. Conductivities were equated to those of standard potassium chloride solutions and adjusted for dilution.

The results showed that by the 7th day, the ionic strength of the heated plants was only 73% of that of the controls (Figure 21; statistical analysis in Appendix 10). There was no significant effect of infection.

The extent of cell enlargement in the heated plants grown in solution culture was much less than that for plants grown in UC mix. The ionic strength of sap from heated plants grown in the normal way is therefore

FIGURE 21.

The ionic strength of sap extracts from healthy and TAV-infected tobacco plants growing in solution culture after varying periods of heat treatment compared with that of extracts from unheated plants.


1·35
1·25
1·15
1·05
0·95
IONIC STRENGTH (as % KCl, F.W.)
unheated
heat treated
0
1
3
5
7
DAYS AFTER COMMENCING HEAT TREATMENT

probably much less than is indicated by the results presented here. A pilot investigation of the reason for the difference in form between heated plants grown in solution and soil culture revealed that the form of heated plants in solution culture could be made to approach that observed in soil culture if the degree of aeration of the culture solutions was reduced.

DISCUSSION.

Plants have been cured from many different virus infections by subjecting them to heat treatment. The mechanisms whereby therapy is achieved have not been investigated previously in any great detail as workers have concentrated on the practical aspects of obtaining virus-free material for distribution and cultivation. Overall, the effect must be that heat treatment is successful when it induces the rate of viral degradation to proceed more rapidly than that of synthesis (Kassanis & Posnette, 1961). My results indicate that heat treatment induces both a decreased rate of viral synthesis and an increased rate of viral degradation compared with the rates of these two activities in plants grown at normal temperatures.

The decreased rate of viral synthesis during heat treatment appears to reflect the incapacity of viral RNA to compete successfully with host messenger ribonucleic acids on which to synthesise their respective proteins. The degree of competition between viral and host messenger RNA for ribosomes increases greatly in heat-treated plants because the rate of host protein synthesis increases and because the concentration of ribosomes decreases in plants at 36^{o}C.

The increased rate of viral degradation during heat treatment is not due solely to a direct effect of temperature as the kinetics of the inactivation are greater than one of the 1st order. Heat induced stress causes an increase in the concentration of ribonucleases and

polyphenoloxidases within the leaf tissue. These enzymes and their products, respectively, may directly inactivate plant viruses and the evidence presented indicates that these two factors have a major role in increasing the rate of viral inactivation at high temperature. In addition, the kinin:auxin ratio falls markedly in heated plants resulting in gross cell enlargement and hence a decline in the ionic strength of the leaf sap. This change, together with an observed decline in the pH of the sap in heated plants, may confer additional instability upon viruses.

These results were obtained using a virus, isolated from chrysanthemum, cultured in the systemic host Nicotiana tabacum var. Hickory Prior. The virus isolate (TAV) was a strain of tomato aspermy virus, on the basis of symptoms and host range as described by several workers (Blencowe & Caldwell, 1949; Govier, 1957; Grogan et al., 1963; Hollings, 1955; Lawson, 1967; Smith, 1957). The physical properties of the isolate also agreed with those recorded for the virus (Gibbs, 1969), and it was readily amenable to heat treatment. TAV appeared to be serologically identical with Victorian and Californian isolates of the virus, but did not appear to be serologically related to English isolates. Mink (1969) recently found that American aspermy isolates also failed to react with antisera developed against English isolates of the virus. It may be that these differences are more apparent than real as van Regenmortel (1966) has pointed out that spurious

results can be obtained if serological tests are not done with balanced levels of antigen and antiserum. I am uncertain whether this was done in my serological tests between TAV and the English antiserum.

I made no attempt to obtain a pure strain of the virus by working with isolates obtained from single local lesions. Figures quoted by Price (1964) on mutation rates of plant viruses indicate that the particles derived from a single lesion would soon contain many variants. The strategem in this work was to transfer TAV to young tobacco plants at frequent intervals with inoculum derived from several plants so that there was a continual selection for that combination of TAV forms which multiplied most rapidly and survived best in that host. There was no indication that the form combination of TAV changed during the three years that it was used. This supports the views of Bawden (1964b) who suggested that virus cultures remain stable under a constant host-environment regime because natural selection operates against the infinitesimal possibility that any newly-formed variants would be better adapted to predominate over those of their parents from which they were derived.

The experimental results obtained using TAV in this work may be classified into three major groups. They are those concerning virus culture and assay (sections I-IV), those concerning virus synthesis at high temperature (sections V-XI), and those concerned with viral degradation at high temperature (sections XII-XVII).

The early experimental work was aimed at developing a reliable and sensitive assay procedure so that low virus concentrations in heat-treated plants could be measured. There are two major difficulties associated with reliably measuring the concentration of infective virus within plants. The first problem arises with the extraction of virus from the plant tissue. Conventional methods of trituration followed by expression through cloth may be very inefficient in extracting virus. Hollings et al. (1968) found that far more cucumber mosaic virus remained attached to the plant fibre and debris following trituration than was present in the filtered sap extract. Therefore, it was necessary to assume that the various treatments applied to infected plants in this work did not alter the efficiency with which TAV was extracted from them, and it is difficult to envisage why this should not have been so. Harrison (1956) had to make similar assumptions during his studies on the effect of temperature on the rate of tobacco necrosis virus multiplication in French bean leaves.

The second problem was the possibility that heat, and other treatments employed during this study would alter concentration of inhibitors of infection in extracts from plants, so that apparent changes in virus concentration, as measured by infectivity assays, would be erroneously ascribed to the effect of treatments on virus concentration when in fact they were due to the effect of treatments on the concentration of inhibitors of infection. It was readily

shown that such inhibitors were present in diseased tobacco leaf tissue, but there was no evidence that their concentration was altered by heat treatment. Emulsification of homogenates with chloroform partially removed the inhibitors and this was a useful technique, particularly when assaying low virus concentrations. Ready access to a preparative ultracentrifuge would have assisted greatly in overcoming this problem further as virus purification by differential centrifugation removes additional inhibitor not destroyed by chloroform and also enables concentration of virus. The degree to which chloroform enhanced infectivity depended on the proportion of inoculated and systemically infected leaves in the sample. The inhibitor appeared to be virus-induced and in this respect was similar to the antiviral principle found in tomato plants infected with tobacco mosaic virus (Chadha & MacNeill, 1969a; 1969b). The finding that low speed centrifugation partially removed the inhibitors negated the possibility that the effect of chloroform was on the release of virus attached to membranes and from within chloroplasts, rather than on the inhibitor. The nature of inhibitors in infected leaf extracts was not investigated, nor was the separate factor which interfered with serological tests in agar gel and which was heat-labile.

Another aspect of the assay procedure studied, after some early experiments had indicated that Chenopodium amaranticolor was the most sensitive local lesion host for TAV, was to establish those conditions which maximised the

sensitivity and reliability of this species for measuring the infectivities of TAV inocula.

The texture of the leaves was correlated with their response to inoculation. Leaves on plants grown under conditions of high light intensity and long days were elongated and developed a very glaucous surface texture which made them insensitive to inoculation. A similar, but more pronounced, leaf form occurred on flowering plants and it may have been this factor which made them appear immune, due to failure to bring virus particles in contact with infectible sites below the bloom of the leaf. The factor responsible for this alteration in the leaf character of flowering plants was graft transmissable. Therefore it was necessary to grow these plants under moderately heavy shading in the summer and provide them with supplementary light to increase daylength in the winter to prevent flowering.

Heating the plants at 36^{o}C for 24 hours prior to inoculation decreased their susceptibility. This is in contrast to the results of Kassanis (1952) with several other virus-host combinations. Pre-inoculation treatments act by altering the number of ectodesmata (Brants, 1964) and there is no reason to suppose that all species should respond similarly, just as flowering response is affected differently by daylength and temperature. In fact, all the plants which Kassanis (1952) found responding more sensitively to inoculation following heat treatment flower indeterminately whereas C. amaranticolor is a short day plant.

The other pre-inoculation treatment investigated, that of placement in the dark for 24 hours, greatly increased the susceptibility of C. amaranticolor to TAV. This agrees with the findings of Bawden & Roberts (1947) for several other plant virus combinations. However, the effect is not universal, and appears to be confounded with others as Matthews (1953) found that plants inoculated early in the day were less susceptible than those inoculated in the afternoon. Also, Helms & McIntyre (1967) found that the susceptibility of bean leaves to inoculation with tobacco mosaic virus increased with time of illumination following a dark treatment.

Results similar to those of Helms & McIntyre were obtained with the C. amaranticolor - TAV combination which appeared to contradict those on the effect of a pre-inoculation dark treatment. However, further investigation established that the apparent increase in susceptibility with time after illumination following a dark treatment was not an effect of light, but rather that the susceptibility of one half of a leaf was increased by prior injury to the opposite half. Nienhaus & Yarwood (1963) and Yarwood et al. (1962) reported that using heat as the form of injury also increased the susceptibility of bean leaves to later inoculations with different viruses. Their responses to injury were as rapid and even more marked than those reported here, and they suggested that the effects were due to the translocation of a wound hormone. In C. amaranticolor the changes in

susceptibility to TAV were correlated with peroxidase concentration in the leaf halves. However, this result does not prove necessarily that the changed susceptibility of the leaves was due to their altered peroxidase level because although Farkas et al. (1960) implicated oxidising enzymes in local lesion development, more recent evidence, reviewed by White (1968), is inconsistent with this hypothesis. However, they do indicate that rapid changes occur within plants following mild injury which are not confined to the injured portions, and these markedly alter subsequent susceptibility to infection. Another instance of rapid changes in the enzyme content of homogenates derived from cells soon after injury was reported by Randles (1968) who found that ribonuclease levels increased rapidly in Chinese cabbage leaves following inoculation. It is difficult to envisage leaves producing and translocating enzymes so rapidly following injury, and it may be that these increases result from the injury-stimulated rupture of lysozomes releasing lytic enzymes into the cytoplasm. Work in Lwoff's laboratory (Lwoff, 1969) has shown that thermal shock destroys virus in polio-infected cells due to the release of ribonucleases, which inactivate the virus, from lysozomes into the cytoplasm.

After a satisfactory assay procedure had been developed, it was readily established that the concentration of TAV declined in plants during heat treatment, whereas the virus accumulated in the leaves of inoculated plants grown

in the glasshouse. Net changes in the absolute protein content of both healthy and infected plants in the two environments followed similar patterns. However, the extent to which differential changes in the rates of synthesis and degradation of TAV and plant proteins was responsible for the altered accumulation rates at high temperature necessitated short term pulse-chase experiments to measure the rate of one of these factors, and by deduction, the rate of the other. The possibility of doing such experiments with TAV seemed fraught with difficulties because of the very low concentration of spherical viruses found within plant cells (Bawden, 1964a), and due to the lack of ready access to a preparative ultracentrifuge to separate and concentrate TAV from normal host constituents. Therefore experiments were designed to measure the rate of host protein synthesis in heated and unheated plants because the mechanism of host protein synthesis closely allies that of viral synthesis (Bosch et al., 1966; Weissmann et al., 1966).

Measurement of the true rate of overall protein synthesis in a plant is extremely difficult. Plant proteins are in a continual state of flux, constantly being synthesised and destroyed, and individual turnover rates vary greatly (Dorner et al., 1957; Hellebust & Bidwell, 1964; Racusen & Foote, 1960). The work reported in this thesis indicates that high temperature has a differential action on the rate of synthesis and breakdown of individual proteins, because the concentration of some enzymes, such as polyphenoloxidases

and ribonucleases, increased while the total protein content of plants fell during heat treatment. Also, there was a large difference between the rate of loss of the chloroplast and of cytoplasmic proteins as observed in the analytical ultracentrifuge.

The use of a single labelled amino acid to measure the overall rate of protein synthesis may produce very misleading results if the proportion of the amino acid in those proteins whose concentration rises during heat treatment differs from that in the proteins whose concentration falls during heat treatment. Plant proteins contain generally low, but very variable, proportions of the sulphur containing amino acids (Lugg & Weller, 1944) and therefore the results obtained in this work using $^{35}SO_4^=$ as a precursor to measure the relative rates of protein synthesis in heated and unheated plants may be fallacious.

On the other hand, the supply of a "balanced" mixture of labelled amino acids would yield little information if heat treatment reduces protein synthesis, because the synthesis of one or more individual amino acids supplied in the incubation medium is normally blocked or limiting. Langridge (1963), in reviewing work on biochemical aspects of temperature response, listed 14 amino acids which behaved as 'high temperature lesions' in different strains of bacteria, yeasts and fungi grown at high temperature. However, the fact that tobacco plants did increase in dry weight at 36^{o}C and that some proteins accumulated rapidly

indicate it is most unlikely that any temperature lesions involving amino acids developed in the work reported here. Therefore, the use of ^{14}C Chlorella protein hydrolysate to record the overall rate of protein synthesis, whereby the plant tissue is supplied with the whole range of amino acids in approximately natural proportions, was considered to produce the best measure of the true situation occurring in heated and unheated tobacco plants, viz. that the absolute plant protein content falls during heat treatment, due to a far greater increase in the rate of breakdown than the increase in rate of synthesis.

The rate of virus degradation, as with overall host protein breakdown, likewise appeared to increase in heated plants. However, the increased rate of degradation was not due solely to an effect of high temperature on the virus. This was indicated by the fact that the rate of TAV inactivation in extracts held at normal temperature was greater in those from heated than from unheated plants. Also, the proportion of cures of infected plants was often greater for shorter rather than longer periods of heat treatment, both in this work, and in that reported by Welsh & Nyland (1965); similar information can be extracted from the data of Posnette et al., (1963). Likewise, some earlier work of mine, mentioned by Stubbs (1966), on heat treatment of apple trees, indicated that healthy material could be obtained by propagating from vegetative growths developing after heat treatment, whereas propagules taken from growths formed during the period of

treatment were not virus-free. It seems that high temperature induces changes in the host which accelerate the rate of virus degradation. Direct evidence for this came from the dilution heating experiments, conducted both 'in vivo' and 'in vitro', which established that inactivation proceeds according to a second or higher order interaction.

Some clues as to possible factors involved in a host-mediated acceleration of viral degradation during heat treatment came from some early experiments aimed at determining the optimum constitution of the homogenising medium used to extract TAV from tobacco for assay on to C. amaranticolor. These included the pH, molarity, and concentration of reducing agents in the homogenising medium.

The pH of the extracting buffer was critical for efficient transmission. This prompted an examination of the pH of sap extracted from plants after varying periods of heat treatment. The hydrogen ion concentration increased markedly during heat treatment. The fact that TAV is most infective at pH 7.5 does not indicate necessarily that the virus is most stable at this value. One of the chief aims of fixing the pH of an homogenising medium is to adjust the surface charge of the virus particles so that their affinity for the proteinaceous infectible sites (Wu & Rappaport, 1961), situated in ectodesmata (Brants, 1964) and broken leaf trichomes (Kontaxis & Schlegel, 1962), is maximised. Therefore, there may be little correlation between the infectivity and stability of TAV in leaf homogenates. For

example, tomato aspermy virus is relatively stable at pH 4.6 (Grogan et al., 1963) but is not infective under these conditions.

The molarity of the extracting buffer is critical for efficient transmission. The fact that such a high molarity buffer was necessary probably partially reflected the necessity to raise the pH of infected plant extracts to that value at which TAV was most infective. The reason why infectivity was greater in buffer made from sodium rather than potassium salts was not investigated. Perhaps it was due to an effect on the integrity of some cellular organelle, such as lysozomes, whose contents would lead to virus inactivation. Thornberry (1935) compared the infectivities of tobacco mosaic virus inocula extracted in the presence of Na, K, and NH_4 dibasic phosphates and, although he found no significant difference between the three, there was a trend in the figures with lesion numbers being greatest for Na, followed by K and then NH_4. Allard (1916) more than doubled the infectivity of a tobacco mosaic extract by adding Na_2SO_4 to a final concentration of 0.023 molar. Yarwood (personal communication) also increased the infectivity of virus preparations by addition of either NaCl or Na_2SO_4 but he had assumed that these effects were due to changes in the molarity of the inocula.

These findings on the effect of buffer molarity on infectivity, together with the observations on changes on the ratio of fresh weight : dry weight in heated plants,

prompted an investigation of ionic strength of sap extracted from plants after varying periods of heat treatment. The ratio of the ionic strength of the sap from heated to that of unheated plants declined markedly with time of treatment.

The significance of the changes in the pH and ionic strength of sap extracted from plants undergoing heat treatment is not easily assessed. There are many reports on changes in the stability of purified plant virus preparations 'in vitro' with alterations in pH and ionic strength of the suspending medium (e.g. Bancroft et al., 1967; Gibbs, 1969; Incardona & Kaesberg, 1964; Scott, 1963). It is difficult to gauge the significance of these results in relation to my data as they were carried out within ranges of pH and ionic strength which are not encountered 'in vivo'. However, they do indicate that the stability of viruses may vary with these factors and it is interesting that these reports dealt with viruses closely related to those amenable to heat treatment.

In view of the fact that the commonly prevailing strains of viruses have evolved within their hosts over long periods of time (Bawden, 1964b), it may not be too speculative to suggest that their stability would be greatest under those conditions normally prevailing within their hosts, and that any induced deviation from this situation would be likely to confer instability. However, this surmise needs verification by investigating the stability of purified TAV preparations in solutions of differing pH and salt concentration.

It seems unlikely, on this basis, that TAV would be most stable at pH 7.5 in 0.3 M salt solutions and in retrospect, experiments which involved comparisons between the behaviour of TAV 'in vivo' and 'in vitro' at high temperature, may have been performed better by treating the extracts in low molarity buffer at about pH 5.0, and adjusting them to those conditions under which TAV is most infective immediately prior to assay. For example, Price (1940) found that the energy of inactivation of tobacco mosaic virus varied markedly with pH, and the findings that RNA was released from turnip yellow mosaic virus when heated 'in vitro' but not 'in vivo' (Lyttleton & Matthews, 1958; Matthews & Lyttleton, 1959) may have been due to a pH effect as their 'in vitro' tests were carried out at neutrality.

Another reason why TAV is most infective at pH values above neutrality may be due to the fact that ribonucleases, which were found to directly inactivate the virus, are relatively inactive at this pH. The concentration of RNase increases in plants during heat treatment, and the decrease in pH of sap extracts from heated plants to about 5.5 approximates the optimum for the activity of this class of enzymes. These findings provide tentative evidence for attributing an important role to this enzyme in the inactivation of virus during heat treatment.

The other factor found to increase the infectivity of TAV in extracts from tobacco plants was the inclusion of reducing agents in the homogenising medium which suggested

that TAV, like many other related spherical viruses, is inactivated by the products resulting from the action of polyphenoloxidase (PPO) enzymes. However, addition of sodium diethyldithiocarbonate (DIECA) to the homogenising medium, which inhibits the activity of this enzyme system by chelating copper, reduced infectivity. Similar results were obtained by Hampton & Fulton (1961) for prune dwarf virus. These incongruities were explained by Mink (1967) who found that DIECA is oxidised by cytochrome oxidase to tetraethylthiuram disulphide which results in a marked increase in the pH of leaf homogenates.

The concentration of PPO increased dramatically over a 15-day period of heat treatment. This rise was inversely correlated with the concentration of its chief substrate, chlorogenic acid (CA), which indicates that CA was being oxidised 'in vivo' during heat treatment. The finding that the rate of inactivation of TAV during heat treatment was reduced by spraying plants with chlororesorcinol, an 'in vivo' inhibitor of PPO, proved that oxidised phenolic compounds have a role in destroying infective virus in plants at $36^{o}C$.

However, the extent to which these two classes of enzymes (PPO and RNase) are responsible for the inactivation has not been determined satisfactorily. Repetition of the experiment described in section XVI, where infectivity changes are followed in varieties of tobacco with differing levels of these enzymes, could prove useful in answering

these questions. In addition, the extent to which TAV and these enzymes may be contained in separate sections of cells, thereby limiting contact with each other, is unknown. However, chlororesorcinol applications to plants undergoing heat treatment reduced the rate of disappearance of TAV, indicating that TAV and PPO must certainly come into contact 'in vivo'. The use of this and other 'in vivo' enzyme inhibitors, if they are available, could aid in solving these problems. The finding that these two enzyme systems may have important roles in inactivation during heat treatment offers possibilities for artificially increasing the rate of inactivation. This was done in one instance through exogenous applications of chlorogenic acid and tyrosinase to plants undergoing heat treatment. Procedures such as these seem worthy of further trial.

Both PPO and RNase are among a group of enzymes whose production is increased by the host under conditions of stress. Therefore, the most desirable temperature used for heat treatment should be expected to vary with the host plant. For example, Campbell (1965) found a temperature of only 34^{o}C was quite satisfactory for eliminating black-currant reversion virus, and this was also the highest temperature that the plants would tolerate. On the other hand, Stubbs (1968) reported that inactivation of citrus exocortis virus in lemon proceeded very slowly at 38^{o}C. This temperature was one at which the lemon trees grew quite satisfactorily, and showed no overt signs of heat stress.

Virus-infection induced stresses, resulting in an increase in the level of these enzymes, may explain also the decline in specific infectivity of spherical viruses in their hosts following attainment of a peak concentration.

Although the concentration of some enzymes, including PPO and RNase, increased in plants during heat treatment, the absolute protein content declined in plants held at 36^{o}C. Therefore, it was somewhat surprising to find that the overall rate of host protein synthesis, measured using ^{14}C Chlorella protein hydrolysate as a substrate, was greater in plants grown in the phytotron cabinet than in the glasshouse. However, homeostatic control mechanisms are the norm in all living organisms and, on this basis, it seems reasonable to expect that the overall rate of synthesis would increase in heated plants whose absolute protein content was decreasing. This situation also applies in detached senescing barley leaves, where the reduction in protein content is partially compensated for by an increase in their rate of protein synthesis (Atkin & Srivastava, 1970). It is difficult to imagine that the TAV concentration in heated plants could be maintained by homeostatic mechanisms, due to the limited amount of genetic material the virus has at its disposal.

The rates at which TAV multiplies in heated and unheated tobacco plants was not established with certainty. This cannot be determined by infectivity assays because of the concurrent synthesis and breakdown of virus particles.

It might possibly be done by subjecting infected plants to a short pulse with a radioactive precursor of some viral component and chasing its incorporation rate as was done for host proteins. However, this presents many practical problems due to the very low concentration of spherical viruses in their host tissues (Bawden, 1964a) and because of the virtual impossibility of achieving complete extraction and separation of virus or viral components from host constituents (Steere, 1959). These points became clear from the experiment aimed at measuring the rate of incorporation of tritiated uridine into TAV RNA in the presence of actinomycin D.

Kummert & Semal (1967) reported a very simple method to measure the rate of multiplication of brome mosaic and tobacco mosaic viruses in host tissue. This involved the collection of acid insoluble material on Millipore filters after emulsification with chloroform following incubation of the tissue in ^{14}C-uridine. However, it is difficult to envisage this method meeting the rigorous requirements needed here, or that it would be generally applicable to all plant viruses, and in particular, to TAV as this virus would not remain intact and soluble in solution with their procedures.

A preferable alternative might be to use a labelled precursor of RNA followed by extraction and separation of the nucleic acids. Several attempts were made to investigate the possibilities of such a procedure using variations on

the method reported by Solymosy et al., (1968) which is based on the use of diethylcarbonate to inhibit nuclease activity during extraction by binding to these, and other, proteins (Muhlrad et al., 1967). However, I obtained very heterogeneous low molecular weight nucleic acid using this method, despite careful sterilisation of all equipment. Apparently my source of diethylcarbonate was ineffective in inhibiting RNase activity as the infectivity of TAV inocula was not increased when they were prepared in its presence.

More satisfactory results might be obtained by employing a phenol-cresol extraction medium to produce undegraded nucleic acids (Kirby, 1965) which could then be very efficiently separated into their various fractions by chromatography on methylated-albumin-kieselguhr columns (Mandell & Hershey, 1960). However, sophisticated fractionating equipment, which was not available, is necessary to apply this method. Despite these difficulties, many observations suggested that the rate of TAV synthesis was very much slower at 36^{o}C than at normal temperatures, and this contention had some tentative quantitative support from the data on the rates of incorporation of labelled uridine into the RNA of infected plants grown at the two temperatures in the presence of actinomycin D.

The mechanism by which most plant viruses are thought to multiply closely parallels that of host protein synthesis (Weissmann et al., 1966; Bosch et al., 1966). They are obligate pathogens, dependent on their hosts for

supply of substrates and cell structures necessary for reproduction. Virus multiplication is therefore in conflict with the host's activities, and the degree of, and outcome of, competition between host and virus for substrates and synthetic sites must therefore be an important factor in determining the virus concentration within infected cells. I propose that the success of virus therapy by heat treatment and/or its aftermath may be at least partially due to an altered balance in the competition between host and virus which favours the host and reduces the rate of virus multiplication. Evidence to support this concept of an alteration in the balance of competition between host and virus for the synthesis of their respective proteins during heat treatment was obtained from experiments designed to investigate changes in virus concentration in plants treated with actinomycin D and kinetin.

Kinetin applications to plants undergoing heat treatment increased the rate at which infective virus declined. The main effect of cytokinins is to stimulate protein and RNA synthesis (e.g. Osborne, 1962). This is due to an increase in the soluble RNA, of which cytokinins are a constituent part (e.g. Hall et al., 1967; Burrows et al., 1969), as the concentration of amino-acyl-t-RNA in the cell controls the rate of translation of m-RNA from DNA templates (Ames & Hartman, 1963; Stent, 1964; Vold & Sypherd, 1968). Application of kinetin to plants undergoing heat treatment was effective in maintaining their protein

concentration and inducing dry weight increases. These responses were due, presumably, to a stimulation of protein synthesis, as kinetin increases the rate of RNA and protein synthesis in tobacco leaf tissue (Parthier & Wollgiehn, 1961). It is postulated that kinetin reduced the rate of virus multiplication in heat-treated plants due to increasing competition from the hosts' stimulated rate of protein synthesis. However, the possibility cannot be excluded that the effect was at least partially due to a stimulated production of substances such as PPO and RNase, which inactivate TAV although Srivistava & Ware (1965) found that kinetin reduced the concentration of RNase in barley leaf tissue, and I could find no evidence for an effect of kinetin on the polyphenoloxidase or chlorogenic acid content of tobacco leaf tissue.

These results agree with those of Kiraly & Pozsar (1964) who found that the rate of accumulation of tobacco mosaic virus in systemically infected tobacco plants grown at normal temperature was reduced by applications of kinetin following infection. They also showed that the rate of virus multiplication was inversely correlated with the rate of host protein synthesis. Cytokinins may also affect establishment of infection (Soans, 1967) if applied before or close to the time of inoculation. This probably accounts for the variable results in several other reports on this subject (see Aldwinckle & Selman, 1967), because their experiments failed to differentiate between effects on

initiation of infection and subsequent virus multiplication. The application of cytokinins to plants undergoing heat treatment, therefore, appears to have a valuable two-fold role. It increases the rate of disappearance of virus and also aids in plant survival. Both of these effects seem to result from a stimulation of plant protein synthesis. Engelbrecht & Mothes (1960) also noted that the heat resistance of Nicotiana rustica was increased by cytokinins.

The characteristics of heat-treated tobacco plants is consistent with a reduced kinin:auxin ratio, e.g. cessation of mitosis, loss of apical dominance, gross cell enlargement, reduction in absolute protein content, senescence, and counteraction of high temperature effects by kinetin. I was unable to obtain direct evidence of a changed ratio, because the method used by Kiraly et al. (1966) for the extraction, purification and assay of naturally occurring cytokinins in beans was not successful with tobacco leaf tissue. Auxin assays were not attempted. The observation of Vine & Jones (1969) that meristem tips taken from hops would only develop roots in culture if the plants had been pre-heat-treated for 2-4 weeks is also suggestive of a decrease in cytokinin content relative to auxin concentration during heat treatment.

There is strong circumstantial evidence that cytokinins are produced in the roots and exert their effect following transport to meristematic tissues (e.g. Burrows & Carr, 1969). Flooding the root system of plants

(deprivation of oxygen) greatly reduces cytokinin production and its subsequent upward movement. In addition, the root system is thought to be the site of oxidative inactivation of excess auxins synthesised in above-ground parts, and auxin accumulates in the shoots of flooded plants (Phillips, 1964a, 1964b). These findings are pertinent because they strengthen the contention that the kinin:auxin ratio was reduced in my container-grown heated plants. When tobaccos were grown at 36°C in solution cultures supplied with continual and vigorous aeration, their appearance was not perceptibly different from that of control plants grown at normal temperature. Simulation of the growth patterns of container grown plants was possible merely by reducing the aeration of the plants in solution culture. This suggests that these altered characteristics of plants at high temperature resulting from a changed hormone regime are at least partly due to the lack of available oxygen in the soil at 36°C.

Notes on methods to increase plant survival during heat treatment support this idea. Posnette & Cropley (1958) found that strawberry plants survived less well in plastic pots, where soil aeration is restricted and temperature higher, than in porous clay pots. Nyland (1964) recommended that watering of heat-treated plants should be kept to the absolute minimum consistent with maintaining turgidity and that plants with large carbohydrate reserves survive better than recently-potted plants.

Posnette (personal communication) has found that hop survival is increased if the roots are maintained at normal temperature and only the shoots allowed to protrude into the heat cabinet. An additional factor is that plants may die during heat treatment if grown in unsterilised soil because many root-rotting pathogens are favoured by the prevailing high temperature and rather anaerobic conditions. Bolton (1967) reported that watering of plants with potassium permanganate solutions overcame this problem. It is not clear, however, whether methods which improve plant survival by increasing soil aeration and thus inducing more normal growth are desirable in terms of virus therapy. This is a point of great practical importance and requires full investigation. An attempt was made to answer this question by assaying the infectivity of plants grown at 36°C in solution cultures aerated to differing degrees. Unfortunately, no lesions developed on the test plants following inoculation from the treated plants.

The second series of experiments supporting the hypothesis that heat treatment results in an altered balance in the competition between virus and host involved the use of actinomycin D. TAV accumulation was greater in plants grown at normal temperatures supplied with low concentrations of actinomycin D. In addition, measurable virus synthesis occurred at high temperature when actinomycin D was applied to the host cells whereas no synthesis could be detected in the absence of the antibiotic. Under some conditions at

least, then, TAV may multiply in tobacco at 36°C.

Actinomycin D at high concentration halts host protein synthesis by binding to DNA and thus inhibiting the synthesis of messenger RNA by DNA-dependent RNA polymerases (Reich & Goldberg, 1964). The antibiotic does not immediately affect viral RNA synthesis (Sanger & Knight, 1963) because this occurs independently of the host's DNA (Weissmann et al., 1966). As a result, any competition between host and viral synthesis is reduced. Several other reports have appeared indicating increased virus concentration in systemically infected hosts grown at normal temperature in the presence of actinomycin D (e.g. Bancroft & Key, 1964; Sander, 1969; Semal, 1967).

The tempting conclusions is that actinomycin D allowed a higher rate of viral replication in the host at 36°C than would have occurred in its absence. However, it cannot be discounted that a similar rate of synthesis also occurred in the control discs but that no net accumulation of virus was detected because of a greater rate of viral inactivation. Indeed, there was a lower apparent concentration of polyphenoloxidase in extracts from plants grown at normal temperatures in the presence of actinomycin D, while Shinde & Santilli (1967) recorded either lower or higher concentrations of ribonuclease in bean leaves dependent on the quantity of applied antibiotic.

Another difficulty in using actinomycin D was that application of concentrations of the antibiotic which halt

host protein synthesis result in a rapid deterioration of the host tissue and hence destruction of those host systems on which the virus relies for its own multiplication. Therefore, measurements on infectivity changes in the presence of this antibiotic probably do not indicate the real situation in untreated plants growing at 36°C, because such experiments must necessarily be extended over a period of several days by which time the host tissue is practically dead. Short term pulse-chase experiments are the obvious answer, but the practical difficulties of these have already been discussed.

The evidence presented points to ribosome concentration as being an important factor in altering the proposed balance of competition between host protein and viral synthesis during heat treatment. The concentration of ribosomes in tobacco plants was reduced by heat treatment, although initially the concentration may increase slightly. The time taken to record a significant reduction varied between experiments. The rate of disappearance of the ribosomes during the heat treatment period was not a simple phenomenon, and the pattern did not fit any simple inactivation reaction, implying that the rates of synthesis and breakdown changed markedly during the treatment period. Schiebel et al. (1969) similarly observed a reduction in the polyribosome concentration of a myxomycete, Physarum polycephalum, subjected to heat shock, and the ability of thermophilic bacteria to grow at high temperatures has been

attributed to the greater thermal stability of their macromolecules, including ribosomes (e.g. Zeikus et al., 1970).

During heat treatment much greater use must be made of the available ribosomes for protein synthesis. This is indicated by the increased ratio of protein synthesis to ribosome concentration. It may be that, under these conditions, viral RNA competes poorly with host messenger RNA for the available ribosomes on which to synthesise their respective proteins. Different nuclear messenger RNA molecules bind to ribosomes with varying intensities (Naora & Kodaira, 1968) and several plant viral RNA's were found to have differing affinities for Escherichia coli ribosomes (van Duin et al., 1968). Binding intensity of nucleic acids to other macromolecules is normally correlated with their content of guanylic and cytidilic acids because of greater possibilities for hydrogen bonding (Moller et al., 1969), and it is interesting to note that most plant viral RNA molecules, which have been chemically analysed, have low collective proportions of these two bases (Knight, 1964). In addition, the nucleic acids of thermophilic bacteria, which are more stable than those from mesophilic species, have a higher content of guanine and cytosine (Stenesh & Holazo, 1967).

Absolute proof of this altered competition concept, with respect to ribosomes, was not obtained. Its verification requires an 'in vitro' biochemical

investigation of the relative binding affinities of viral and host messenger nucleic acids to ribosomes.

The concept of an altered balance in competition between host and virus for their respective synthetic activities may also explain the success of meristem tip culture in obtaining virus-free material. It was thought that this method succeeded because virus particles failed to enter meristematic tissue. However, virus movement from cell to cell takes place by way of plasmodesmata (Bawden, 1964a) and very large numbers of these inter-cell connections are formed during mitosis due to spindle fibres transversing the median plate at metaphase (Clowes & Juniper, 1964; Juniper & Barlow, 1969). Hollings & Stone (1964) and Walkey & Webb (1968) detected carnation mottle and cherry leaf roll virus, respectively, in apical meristems, and culture of these tips regularly produced virus-free material. These observations imply that the conditions within the host cells of growing excised meristem tips favour the disappearance of infective virus in the same way as does the condition of plant tissue during, and subsequent to, heat treatment. Meristem tips grow extremely rapidly on nutrient media normally enriched with organic substances, including cytokinins. Rates of protein synthesis in these tips must be extremely rapid and the possibility that viral synthesis ceases under these conditions due to a competitive effect seems plausible. However, it should be noted that viruses have not always been found in meristem tips (e.g.

Kassanis, 1950).

In addition, meristem-tip culture combined with heat treatment has often produced virus-free material whereas tip culture alone has been unsuccessful (e.g. Stace-Smith & Mellor, 1968). Campbell's (1962) proposal that this latter type of success results from the rate of virus movement lagging behind the rate of shoot elongation seems unrealistic, because the rate of virus movement would increase with temperature due to a higher speed of cytoplasmic streaming. Also, the results on mitotic activity and cytology of tobacco at 36°C cast doubts on whether reported apparent growth during heat treatment is due to cell division. A cursory inspection of meristems from Jonathon and Granny Smith apple trees growing at 36°C also failed to reveal any evidence of mitotic activity. It seems likely that here, again, the cure from infection is achieved due to reasons suggested for the success of heat treatment and tip culture alone.

In conclusion, my results with TAV in tobacco indicate that heat treatment is most likely to be successful when the temperature is such that a moderate amount of stress is induced on the plant. This hypothesis is advanced because stress may stimulate the host to produce enzymes which either directly or indirectly may inactivate viruses, and because it may alter the rates of overall protein synthesis and breakdown in plants so that the balance in competition between the host and virus for ribosomes is increased in favour of the host. The most desirable

temperature for the heat-treatment of any particular virus, therefore, may vary considerably, dependent upon the host species in which the virus is being treated. A direct effect of temperature on viruses 'per se' may possibly have a very minor role in achieving therapy during, and consequent to, heat treatment.

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BIBLIOGRAPHY.

ABBOT, E.V. (1959).- Diseases in relation to variety planting in 1959. Sugar Bull. 37: 272-3, 283-4 (cited by Nyland & Goheen, 1969).

ALDWINCKLE, H.S., and SELMAN, I.W. (1967).- Some effects of supplying benzyladenine to leaves and plants inoculated with viruses. Ann. appl. Biol. 60: 49-58.

ALLARD, H.A. (1916).- Some properties of the virus of the mosaic disease of tobacco. Journ. agr. Res. 6: 649-74.

AMES, B.N., and HARTMAN, P. (1963).- The histidine operon. Cold Spring Harbor Symposium on Quantitative Biology 28: 1348-56.

ANON. (1968).- Plant pathology. E. Malling Res. Sta. Rept. 1967: 31.

ANTOINE, R., and RICAUD, C. (1964).- Cane diseases. Rept. Sugar Ind. Res. Inst. Mauritius, 1963: 77-89 (cited by Hollings, 1965a).

ATKIN, R.K., and SRIVASTAVA, B.I.S. (1970).- Studies on protein synthesis by senescing and kinetin - treated barley leaves. Physiol. Plant. 23: 304-15.

BABOS, P., and KASSANIS, B. (1962).- Unstable variants of tobacco necrosis virus. Virology 18: 206-11.

BABOS, P., and KASSANIS, B. (1963a).- Thermal inactivation of tobacco necrosis virus. Virology 20: 490-7.

BABOS, P., and KASSANIS, B. (1963b).- Serological relationships and some properties of tobacco necrosis virus strains. J. gen. Microbiol. 32: 135-44. (cited by Rees et al., 1970).

BAGI, G., and FARKAS, G.L. (1967).- On the nature of the increase in ribonuclease activity in mechanically damaged tobacco leaf tissues. Phytochemistry 6: 161-9.

BAKER, K.F. (1957).- The UC system for producing healthy container-grown plants. Calif. agric. exp. Stat. Ext. Serv. Manual 23.

BAKER, K.F. (1962).- Thermotherapy of planting material. Phytopathology 52. 1244-55.

BALD, J.G. (1964).- Cytological evidence for the production of plant virus ribonucleic acid in the nucleus. Virology 22: 377-87.

BALD, J.G. (1971).- (unpublished data).

BALD, J.G., and SAMUEL, G. (1934).- Some factors affecting the inactivation rate of the virus of tomato spotted wilt. Ann. appl. Biol. 21: 179-90.

BANCROFT, J.B., HILLS, G.J., and MARKHAM, R. (1967).- A study of the self-assembly process in a small spherical virus. Formation of organised structures from protein subunits 'in vitro'. Virology 31: 354-79.

BANCROFT, J.B., and KEY, J.L. (1964).- Effect of actinomycin D and ethylenediamine tetraacetic acid on the multiplication of a plant virus in etiolated soybean hypocotyls. Nature 202: 729-30.

BAWDEN, F.C. (1941).- The serological reactions of viruses causing tobacco necrosis. Brit. J. exp. Path. 22: 59-70.

BAWDEN, F.C. (1954).- Inhibitors and plant viruses. Advan. Virus Res. 2: 31-57.

BAWDEN, F.C. (1964a).- "Plant viruses and virus diseases". (Ronaldson Press Co., New York).

BAWDEN, F.C. (1964b).- Speculations on the origins and nature of viruses. in "Plant virology". (Ed. M.K. Corbett & H.D. Sisler) (University of Florida Press: Gainesville) pp. 365-85.

BAWDEN, F.C., and ROBERTS, F.M. (1947).- The influence of light intensity on the susceptibility of plants to certain viruses. Ann. Appl. Biol. 34: 286-96.

BELL, A.F. (1933).- A new disease of cane in north Queensland. Queensland Agric. Journ. 40: 460-4. (abs. in Rev. appl. Mycol. 33: 325).

BERG, T.M. (1964).- Studies on poplar mosaic virus and its relation to the host. Mededel. Landbouwhoges-school Wageningen 64: 1-72. (abs. in Rev. appl. Mycol. 44: 550).

BEST, R.J. (1937).- The chemistry of some plant viruses. Australian Chem. Inst. J. and Proc. 4: 375-92.

BLENCOWE, J.W., and CALDWELL, J. (1949).- Aspermy - a new virus disease of tomato. Ann. Appl. Biol. 36: 320-6.

BOARDMAN, N.K., FRANCKI, R.I.B., and WILDMAN, S.G. (1966).- Protein synthesis by cell-free extracts of tobacco leaves. III Comparison of the physical properties and protein synthesizing activities of 70 s chloroplast and 80 s cytoplasmic ribosomes. J. Mol. Biol. 17: 470-87.

BOL, J.F., and VELDSTRA, H. (1969).- Degradation of alfalfa mosaic virus by pancreatic ribonuclease. Virology 37: 74-85.

BOLTON, A.T. (1967).- The inactivation of veinbanding and latent C viruses in strawberries by heat treatment. Can. J. Plant Sci. 47: 375-80.

BONNER, J. (1965).- Ribosomes. in "Plant biochemistry". (Eds. J. Bonner & E.J. Varner) (Academic Press: London) ch. 3.

BOSCH, L., van KNIPPENBERG, P.H., VOORMA, H.O., and van RAVENSWAYCLAASEN, J.C. (1966).- Protein synthesis by cell-free extracts of E. coli programmed with plant viral RNA. in "Viruses of plants". (Ed. A.B.R. Beemster & J. Dijkstra) (North-Holland Publishing Co.: Amsterdam) pp. 275-94.

BRANTS, D.H. (1964).- The susceptibility of tobacco and bean leaves to tobacco mosaic virus infection in relation to the condition of the ectodesmata. Virology 23: 588-94.

BRIERLEY, P. (1955).- Symptoms induced in chrysanthemums on inoculation with viruses of mosaics, aspermy and flower distortion. Phytopathology 45: 2-7.

BRIERLEY, P. (1957).- Virus-free hydrangeas from tip cuttings of heat-treated, ringspot affected stock plants. Plant Disease Reptr. 41: 1005.

BRIERLEY, P. (1964).- Heat cure of carnation viruses - Plant Disease Reptr. 48: 143.

BRIERLEY, P., and SMITH, F.F. (1957).- Symptoms of chrysanthemum flower distortion, dodder transmission of the virus, and heat cure of infected plants. Phytopathology 47: 448-50.

BRIERLEY, P., and SMITH, F.F. (1958).- Some characteristics of eight mosaic and two rosette viruses of chrysanthemum. Plant Disease Reptr. 42: 752-63.

BROADBENT, L. (1965).- The epidemiology of tomato mosaic. XI Seed transmission of TMV. Ann. appl. Biol. 56: 177-205.

BRUEHL, G.W. (1953).- Chlorotic streak disease of Pennisetum purpureum. Plant Disease Reptr. 37: 34-5.

BURROWS, W.J., ARMSTRONG, D.J., SKOOG, F., HECHT, S.M., BOYLE, J.T.A., LEONARD, N.J., and OCCOLOWITZ, J. (1969).- The isolation and identification of two cytokinins from Escherichia coli transfer nucleic acids. Biochemistry (USA) 8: 3071-6.

BURROWS, W.J., and CARR, D.J. (1969).- Effects of flooding the root system of sunflower plants on the cytokinin content in the xylem sap. Physiol. Plant. 22: 1105-12.

CALAVAN, E.C. (1968).- A review of stubborn and greening diseases of citrus. Proc. 4th Conf. Intern. Organ. Citrus Virologists, Italy: 105-17.

CAMPBELL, A.I. (1962).- Apple virus inactivation by heat therapy and tip propagation. Nature 195: 520.

CAMPBELL, A.I. (1965).- The inactivation of black currant reversion virus by heat therapy. Ann. Rept. Long Ashton Agr. Hort. Res. Sta. 1964: 89-92.

CASALICCHIO, G. (1964).- Applicazione della termterapice nel risanemento delle piante di Fico affette da mosaico. Phytopath. Mediterranea 3: 184-5. (abs. in Rev. appl. Mycol. 45: 1873).

CAUDWELL, A. (1966).- L'inhibition 'in vivo' du virus de la flavescence doree par la chaleur. Ann. Epiphyties Suppl. 17: 61-6 (abs. in Rev. appl. Mycol. 46: 1492f).

CHADHA, K.C., and MacNEILL, B.H. (1969a).- An antiviral principle from tomatoes systemically infected with tobacco mosaic virus. Can. J. Botany 47: 513-8.

CHADHA, K.C., and MacNEILL, B.H. (1969b).- Influence of temperature upon levels of virus and an induced antiviral principle in tomato plants systemically infected with tobacco mosaic virus. Can. J. Microbiol. 15: 1469-71.

CHAMBERS, J. (1954).- Heat therapy of virus - infected raspberries. Nature 173: 595-6.

CHAMBERS , J. (1961).- The production and maintenance of virus-free raspberry plants. J. hort. Sci. 36: 48-54.

CHANCE, B., and MAEHLEY, A.C. (1955).- Assay of catalases and peroxidases. in "Methods in enzymology" (Ed. S.P. Colowick & N.O. Kaplan) (Academic Press, New York) Vol. 2 pp. 764-75.

CHANT, S.R. (1959).- A note on the inactivation of mosaic virus in cassava (Manihot utilissima Pohl.) by heat treatment. Empire J. exp. Agric. 27: 55-8.

CHEO, P.C., and POUND, G.S. (1952).- Relation of air temperature, soil temperature, photoperiod and light intensity to the concentration of cucumber virus 1 in spinach. Phytopathology 42: 306-10.

CHIU, R., and SILL, W.H. (1963).- Factors affecting assay of bromegrass mosaic virus on Datura stramonium and Chenopodium hybridum. Phytopathology 53: 69-78.

CHRISTOFF, A. (1958).- Die Obstvirosen in Bulgarien. Phytopathol. Z. 31: 381-436.

CLOSE, R. (1964).- Some effects of other viruses and of temperature on the multiplication of potato virus X. Ann. appl. Biol. 53: 151-64.

CLOWES, F.A.L., and JUNIPER, B.E. (1964).- The fine structure of the quiescent centre and neighbouring tissues in root meristems. J. exp. Bot. 15: 622-30.

COCHRAN, W.C., and COX, G.M. (1957).- "Experimental designs". (John Wiley & Sons. Inc., Sydney) (2nd edn.)

CONVERSE, R.H. (1966).- Effect of heat treatment on the raspberry mosaic complex in Latham red raspberry. Phytopathology 56: 556-9.

DARLINGTON, C.D., and LA COUR, L.F. (1947).- "The handling of chromosomes". 2nd Ed. (George Allen & Unwin Ltd.: London).

DESJARDINS, P.R., WALLACE, J.M., WOLLMAN, E.S.H., and DRAKE, R.J. (1959).- A separation of virus strains from a tristeza-seedling-yellows complex by heat treatment of infected lime seedlings. in "Citrus virus diseases". (Ed. J.M. Wallace) (Agricultural Publications, University of California, Riverside) pp. 91-5.

DIENER, T.O. (1961).- Virus infection and other factors affecting ribonuclease activity of plant leaves. Virology 14: 177-89.

DIENER, T.O., and RAYMER (1967).- Potato spindle tuber virus: a plant virus with properties of a free nucleic acid. Science 158: 378-81.

DIENER, T.O., and WEAVER, M.L. (1959).- Reversible and irreversible inhibition of necrotic ringspot virus in cucumbers by pancreatic ribonuclease. Virology 7: 419-27.

DORNE, B. (1968).- Contribution a l'etude de la structure du virus du tomate bushy-stunt. Thesis, University of Strasbourg. (cited by Bol & Veldstra, 1969).

DORNER, R.W., KAHN, A., and WILDMAN, S.G. (1957).- The proteins of green leaves. VII. Synthesis and decay of cytoplasmic proteins during the life of the tobacco leaf. J. biol. Chem. 229: 945-52.

DOVE, L.D. (1967).- Ribonuclease activity of stressed tomato leaflets. Plant Physiol. 42: 1176-8.

DUCREUX, G. (1963).- Contribution a l'etude comparee des infrastructures de feuilles de vignes saines et court-nouees cultivees in vitro. Naturalia monspel. Ser. bot. 15: 53-72. (abs. in Rev. appl. Mycol. 44: 2324).

DUIN, J. van, PLEY, C.W., BONNET-SMITS, E.M., and BOSCH, L. (1968).- Interaction between plant viral RNA and Escherichia coli ribosomes. Biochim. Biophys. Acta 155: 444-55.

EHLERS, C.G. (1957).- Separation and identification of viruses that incite disease of stone fruits. Dissertation Abstr. 17: 1879-80.

EL-BANNA, M.T., MOURSI, M.A., and NOUREL-DIN, F. (1967).- Studies on sugar cane ratoon stunting virus disease. Agric. Res. Rev. Cairo 45: 74-100.

ENGELBRECHT, L., and MOTHES, K. (1960).- Kinetin als faktor des hitzresistenz. Ber. Deut. Botan. Ges. 73: 246-57.

ELLENBERGER, C.E. (1960).- Heat inactivation of some viruses in plum varieties and rootstocks. E. Malling Res. Sta. Rept. 1959: 99-101.

FARKAS, G.L., KIRALY, Z., and SOLYMOSY, F. (1960).- Role of oxidative metabolism in the localisation of plant viruses. Virology 12: 408-21.

FENTON, T. (1969).- Production of chrysanthemum stock free from aspermy virus. Ministry of Agriculture, Fisheries & Food; National Agricultural Advisory Service; Experimental Husbandry Farms & Experimental Horticultural Stations; 10th progress report. pp. 98-9.

FERNOW, K.H., PETERSON, L.C., and PLAISTED, R.L. (1962).- Thermotherapy of potato leafroll. Am. Potato J. 39: 445-51.

FISHER, R.A., and YATES, F. (1963).- "Statistical tables for biological, agricultural and medical research". (Oliver and Boyd, London) (6th edn.).

FITZPATRICK, R.E., STACE-SMITH, R., and MELLOR, F.C. (1954).- Heat inactivation of some strawberry and raspberry viruses. Proc. Can. Phytopathol. Soc. 22: 13. (abs. in Rev. appl. Mycol. 35: 109).

FRANCKI, R.I.B. (1968).- Inactivation of cucumber mosaic virus (Q strain) nucleoprotein by pancreatic ribonuclease. Virology 34: 694-700.

FRANCKI, R.I.B., RANDLES, J.W., CHAMBERS, T.C., and WILSON, S.B. (1966).- Some properties of purified cucumber mosaic virus (Q strain). Virology 28: 729-41.

FRAZIER, N.W., VOTH, V., and BRINGHURST, R.S. (1965).- Inactivation of two strawberry viruses in plants grown in a natural high temperature environment. Phytopathology 55: 1203-5.

FRISCH-NIGGENMEYER, W., and REDDI, K. (1967).- Studies on ribonuclease in tobacco leaves. I. Purification and properties. Biochim. Biophys. Acta 26: 40-6.

FROSHEISER, F.L. (1969).- Freeing alfalfa clones from alfalfa mosaic virus by heat treatment. Phytopathology 59: 391-2.

FULTON, J.P. (1954).- Heat treatments of virus-infected strawberry plants. Plant Dis. Reptr. 38: 147-9.

FULTON, R.W. (1949).- Virus concentration in plants acquiring tolerance to tobacco streak. Phytopathology 39: 231-43.

FULTON, R.W. (1968).- Serology of viruses causing cherry necrotic ringspot, plum line pattern, rose mosaic and apple mosaic. Phytopathology 58: 635-8.

FURUMOTO, W.A., and MICKEY, R. (1967a).- A mathematical model for the infectivity-dilution curve of tobacco mosaic virus: theoretical considerations. Virology 32: 216-23.

FURUMOTO, W.A., and MICKEY, R. (1967b).- A mathematical model for the infectivity dilution curve of tobacco mosaic virus: experimental tests. Virology 32: 224-33.

GALZY, R. (1966).- Action de la temperature 35°C sur Vitis rupestris atteint de court-noue. Bull. Soc. franc. Physiol. Vegetale 12: 391-9. (cited by Nyland & Goheen, 1969).

GANDY, D.G., and HOLLINGS, M. (1962).- Dieback of mushrooms: a disease associated with a virus. Rep. Glasshouse Crops Res. Inst. 1961: 103-8.

GHABRIAL, S.A., and PIRONE, T.P. (1967).- Physiology of tobacco etch virus-induced wilt of Tabasco peppers. Virology 31: 154-62.

GIBBS, A. (1969).- Plant virus classification. Advan. Virus Res. 14: 263-328.

GIERER, (1957).- Structure and biological function of ribonucleic acid from tobacco mosaic virus. Nature 179: 1297-9.

GILLASPIE, A.G., and BANCROFT, J.B. (1965).- The rate of accumulation, specific infectivity, and electrophoretic characteristics of bean pod mottle virus in bean and soybean. Phytopathology 55: 906-8.

GINOZA, W. (1958).- Kinetics of heat activation of ribonucleic acid of tobacco mosaic virus. Nature 181: 958-61.

GLAZEWSKA, Z. (1963).- Proby zwalczania lisciozwoju chmielu metoda termoterapia. Biul. Inst. Ochr. Rosl., Poznan 1963: 131-4. (abs. in Rev. appl. Mycol. 43: 1370).

GOODMAN, R.N., KIRALY, Z., and ZAITLIN, M. (1967).- "The biochemistry and physiology of infectious plant disease". (D. Van Nostrand Co., Inc.: Princeton). ch. 6.

GOVIER, D.A. (1957).- The properties of tomato aspermy virus and its relationship with cucumber mosaic virus. Ann. appl. Biol. 45: 62-73.

GRANADOS, R.R., and CHAPMAN, R.K. (1968).- Heat inactivation and interactions of four aster yellows virus strains in their vector Macrosteles fascifrons (St.). Virology 36: 333-42.

GRANT , T.J. (1957).- Effect of heat treatments on tristeza and psorosis viruses in citrus. Plant Disease Reptr. 41: 232-4.

GREGORY, F.G., and SEN, P.K. (1937).- Physiological studies in plant nutrition. VI The relation of respiration rate to the carbohydrate and nitrogen metabolism of the barley leaf as determined by nitrogen and potassium deficiency. Ann. Bot. N.S. 1: 521-61.

GRINSTED, J. (1969).- Temperature dependence of RNA breakdown in a thermophilic Bacillus. Biochim. Biophys. Acta 182: 248-9.

GROGAN, R.G., UYEMOTO, J.K., and KIMBLE, K.A. (1963).- Evidence that tomato aspermy and cucumber mosaic viruses are serologically unrelated. Virology 21: 36-42.

HAKKAART, R.A., and QUAK, F. (1964).- Effect of heat treatment of young plants on freeing chrysanthemums from virus B by means of meristem culture. Neth. J. Plant Pathol. 70: 154-7.

HALL, R.H., OSONKA, L., DAVID, H., and McLANNAN, B. (1967).- Cytokinins in the soluble RNA of plant tissues. Science N.Y. 156: 69-71.

HAMID, A., and LOCKE, S.B. (1961).- Heat inactivation of leafroll virus in potato tuber tissues. Am. Potato J. 38: 304-10.

HAMPTON, R.E., and FULTON, R.W. (1961).- The relation of polyphenol oxidase to instability 'in vitro' of prune dwarf and sour cherry necrotic ringspot viruses. Virology 13: 44-52.

HARRISON, B.D. (1956).- Studies on the effect of temperature on virus multiplication in inoculated leaves. Ann. appl. Biol. 44: 215-26.

HARRISON, B.D., and PIERPOINT, W.S. (1963).- The relation of polyphenoloxidase in leaf extracts to the instability of cucumber mosaic and other plant viruses. J. gen. Microbiol. 32: 417-27.

HAYHOE, F.G.H., and QUAGLINE, D. (1965).- Autoradiographic investigations of RNA and DNA metabolism of human lencocytes cultured in phytohaemagglutinin; uridine -5-^{3}H as a specific precursor of RNA. Nature 205: 151-4.

HELLEBUST, J.A., and BIDWELL, R.G.S. (1964).- Protein turnover in attached wheat and tobacco leaves. Can. J. Bot. 42: 1-12.

HELMS, K., and McINTYRE, G.A. (1967).- Light induced susceptibility of Phaseolus vulgaris L. to tobacco mosaic virus infection. I Effect of light intensity, temperature and preinoculation dark period. Virology 31: 191-6.

HERBERG, R.J. (1960).- Determination of carbon -14 and tritium in blood and other whole tissues. Liquid scintillation counting of tissues. Analyt. Chem. 32: 42-6.

HEWITT, E.J. (1966).- "Sand and water culture methods used in the study of plant nutrition". (C.A.B. Tech. Comm. No. 22) (2nd edition, revised).

HILDEBRAND, E.M. (1941).- Rapid transmission of yellow-red virosis in peach. Contrib. Boyce Thompson Inst. 11: 485-96 (abs. in Rev. appl. Mycol. 20: 540).

HILDEBRAND, E.M. (1964).- Heat treatment for eliminating internal cork viruses from sweet potato plants. Plant Disease Reptr. 48: 356-8.

HIRAI, A., and WILDMAN, S.G. (1967).- Similarity in symptoms produced in tobacco plants by actinomycin D and TMV. Virology 31: 721-2.

HITCHBORN, J.H. (1956).- The effect of temperature on infection with strains of cucumber mosaic virus. Ann. appl. Biol. 44: 590-8.

HITCHBORN, J.H. (1957).- The effect of high temperature on the multiplication of two strains of tobacco ringspot virus. Virology 3: 243-4.

HITCHBORN, J.H. (1968).- Evidence for the release of 28 s RNA from turnip yellow mosaic virus heated 'in vitro'. J. gen. Virol. 3: 137-40.

HODGMAN, C.H. (1944).- Solubility of gases in water. in "Handbook of chemistry and physics". (Chemical Rubber Publishing Co., Cleveland, Ohio) (28th edn.) pp. 1340-1.

HOLLINGS, M. (1955).- Investigation of chrysanthemum viruses. I Aspermy flower distortion. Ann. appl. Biol. 43: 86-102.

HOLLINGS, M. (1961).- Virology. Glasshouse Crops Res. Inst. Rept. 1960: 72-8.

HOLLINGS, M. (1962a).- Studies of pelargonium leaf curl virus. I Host range, transmission and properties 'in vitro'. Ann. appl. Biol. 50: 189-202.

HOLLINGS, M. (1962b).- Heat treatment in the production of virus-free ornamental plants. G. Brit. Natl. Agr. Advis. Serv. Quart. Rev. 57: 31-4.

HOLLINGS, M. (1963a).- Virology. Glasshouse Crops Res. Inst. Rept. 1962: 86-93.

HOLLINGS, M. (1963b).- Cucumber stunt mottle, a disease caused by a strain of arabis mosaic virus. J. hort. Sci. 38: 138-49.

HOLLINGS, M. (1964).- Virology. Glasshouse Crops Res. Inst. Rept. 1963: 87-92.

HOLLINGS, M. (1965a).- Disease control through virus-free stock - Ann. Rev. Phytopath. 3: 367-96.

HOLLINGS, M. (1965b).- Virology. Glasshouse Crops Res. Inst. Rept. 1964: 87-97.

HOLLINGS, M. (1965c).- Anemone necrosis, a disease caused by a strain of tobacco ringspot virus. Ann. appl. Biol. 55: 447-57.

HOLLINGS, M. (1965d).- Some properties of celery yellow vein, a virus serologically related to tomato black ring virus. Ann. appl. Biol. 55: 459-70.

HOLLINGS, M., and KASSANIS, B. (1957).- The cure of chrysanthemums from some virus diseases by heat. J. Roy. Hort. Soc. 82: 339-42.

HOLLINGS, M., and NARIANI, T.K. (1966).- Some properties of clover yellow vein, a virus from Trifolium repens L.. Ann. appl. Biol. 56: 99-109.

HOLLINGS, M., and STONE, O.M. (1962).- The attenuation of carnation mottle virus in plants. Rep. Glasshouse Crops Res. Inst. 1961: 100-2.

HOLLINGS, M., and STONE, O.M. (1963).- Turnip crinkle virus isolated from an ornamental variegated cabbage (Brassica oleracea var. capitata L.). Rep. Glasshouse Crops Res. Inst. 1962: 118-25.

HOLLINGS, M., and STONE, O.M. (1964).- Investigation of carnation viruses. I Carnation mottle. Ann. appl. Biol. 53: 103-18.

HOLLINGS, M., and STONE, O.M. (1965a).- Investigation of carnation viruses. II Carnation ringspot. Ann. appl. Biol. 56: 73-86.

HOLLINGS, M., and STONE, O.M. (1965b).- Studies of pelargonium leaf curl virus. II Relationship to tomato bushy stunt and other viruses. Ann. appl. Biol. 56: 87-98.

HOLLINGS, M., and STONE, O.M. (1968).- Techniques and problems in the production of virus-tested planting material. Sci. Hort. 20: 57-72.

HOLLINGS, M., STONE, O.M., and BRUNT, A.A. (1968).- Cucumber mosaic virus. Rep. Glasshouse Crops Res. Inst. 1967: 94-107.

HOLMES, F.O. (1934).- A masked strain of tobacco mosaic virus. Phytopathology 24: 845-73.

HOLMES, F.O. (1960).- Rose mosaic cured by heat treatment. Plant Disease Reptr. 44: 46-7.

HOLMES, F.O. (1965).- Elimination of turnip mosaic virus from a stock of horseradish. Phytopathology 55: 530-2.

HOUTEN, J.G. ten, QUAK, F., and MEER, F.A. van der (1968).- Heat treatment and meristem culture for the production of virus-free plant material. World Rev. Pest Control 7: 115-120.

HOUTMAN, P.W. (1925).- Warmwaterbehandeling van zeefvatenziekte DI 52 bibit, in het groot toegepast. Arch. Suikerind, Ned. - Ind. 33: 631-42.

HUNTER, J.A., CHAMBERLAIN, E.E., and ATKINSON, J.D. (1959).- Note on a modification for inactivating apple mosaic virus in apple wood by heat treatment. New Zealand J. Agr. Res. 2: 945-6.

HUTCHINS, L.M., and RUE, J.L. (1939).- Promising results of heat treatment for inactivation of phony disease in dormant peach nursery trees. Phytopathology 29: 12 (abs.).

INCARDONA, M.L. and KAESBERG, P. (1964).- A pH induced structural change in bromegrass mosaic virus. Biophys. J. 4: 11-21.

IZADPANAH, K., and SHEPHERD, R.J. (1966).- Purification and properties of the pea enation mosaic virus. Virology 28: 463-76.

JENSEN, D.D. (1968).- Influence of high temperature on the pathogenicity and survival of western-X in leaf hoppers. Virology 36: 662-7.

JOHNSON, J. (1926).- The attenuation of plant viruses and the inactivating influence of oxygen. Science 64: 210.

JOHNSON, J. (1947).- Virus attenuation and the separation of strains by specific hosts. Phytopathology 37: 822-37.

JONES, O.P., and VINE, S.J. (1968).- The culture of gooseberry shoot tips for eliminating virus. J. hort. Sci. 43: 289-92.

JOSHI, L., and HOLMES, F.O. (1968).- Induced tolerance of tobacco mosaic virus to heat. Phytopathology 58: 60-1.

JUNIPER, B.E., and BARLOW, P.W. (1969).- The distribution of plasmodesmata in the root tip of maize. Planta (Berl.) 89: 352-60.

KAPER, J.M., and JENIFER, F.G. (1968).- Studies on the interaction of p- mercuribenzoate with turnip yellow mosaic virus. V Induced ribonuclease sensitivity and degradation of the virion. Virology 35: 71-81.

KASSANIS, B. (1950).- Heat inactivation of leaf-roll virus in potato tubers. Ann. appl. Biol. 37: 339-41.

KASSANIS, B. (1952).- Some effects of high temperature on the susceptibility of plants to infection with viruses. Ann. appl. Biol. 39: 358-69.

KASSANIS, B. (1954).- Heat therapy of virus infected plants. Ann. appl. Biol. 41: 470-4.

KASSANIS, B. (1955).- Some properties of four viruses isolated from carnation plants. Ann. appl. Biol. 43: 103-13.

KASSANIS, B. (1957a).- Effects of changing temperature on plant virus diseases - Advan. Virus Res. 4: 221-41.

KASSANIS, B. (1957b).- Some effects of varying temperature on the quality and quantity of tobacco mosaic virus in infected plants. Virology 4: 187-99.

KASSANIS, B. (1967).- Therapy of virus infected plants. Jl Roy. agric. Soc. 126: 105-14.

KASSANIS, B., and LEBEURIER, G. (1969).- The behaviour of tomato bushy stunt virus and bromegrass mosaic virus at different temperatures in vivo and in vitro. J. gen. Virol. 4: 385-95.

KASSANIS, B., and POSNETTE, A.F. (1961).- Thermotherapy of virus-infected plants. Recent advances in Botany 1: 557-63.

KEGLER, H. (1959).- Untersuchungen uber virosen der kernobstes I. Das apfelmosaikvirus. Phytopathol. Z. 37: 170-86.

KEGLER, H. (1967).- Eine einfache Apparatur zur Warmebehandlung viruskranker Obstpflanzen. Arch. Gartenbau 15: 69-74. (cited by Nyland & Goheen, 1969).

KIRALY, Z., and POZSAR, B.I. (1964).- On the inhibition of TMV production by kinetin and adenine in intact tobacco leaves. in "Host-parasite relations in plant pathology." (Ed. Z. Kiraly & G. Ubrizsy) (Research Institute for Plant Protection Budapest, Hungary) pp. 61-4.

KIRALY, Z., POZSAR, B.I., and EL HAMMADY, M. (1966).- Cytokinin activity in rust-infected plants; juvenility and senescence in diseased leaf tissues. Acta Phytopath. Acad. Sci. Hung. 1: 29-37.

KIRBY, K.S. (1965).- Isolation and characterisation of ribosomal ribonucleic acid. Biochem. J. 96: 266-9.

KLECZKOWSKI, A. (1949).- The transformation of local lesion counts for statistical analysis. Ann. appl. Biol. 36: 139-52.

KLECZKOWSKI, A. (1950).- Interpreting relationships between the concentration of plant viruses and numbers of local lesions. J. gen. Microbiol. 4: 53-69.

KNIGHT, C.A. (1964).- Structural biochemistry of plant viruses. in "Plant virology". (Ed. M.K. Corbett & H.D. Sisler) (University of Florida Press: Gainesville) pp. 292-314.

KODAMA, T., and BANCROFT, J.B. (1964).- Some properties of infectious ribonucleic acid from broad bean mottle virus. Virology 22: 23-32.

KONTAXIS, D.G., and SCHLEGEL, D.E. (1962).- Basal septa of broken trichomes in Nicotiana as possible infection sites for tobacco mosaic virus. Virology 16: 244-7.

KRISTENSEN, H.R., and THOMSEN, A. (1958).- Chrysanthemum-viroser. Tidsskr. Planteavl. 62: 627-9 (abs. in Rev. appl. Mycol. 38: 409-10).

KUHN, C.W. (1965).- Decline of specific infectivity of cowpea chlorotic mottle virus in vivo. Virology 25: 9-14.

KUHN, C.W., and BANCROFT, J.B. (1961).- Concentration and specific infectivity changes of alfalfa mosaic virus during systemic infection. Virology 15: 281-8.

KULKARNI, H.Y. (1969).- Transmission of the pathogen of molasses dwarf by Malaxodes farinosus. Phytopathology 59: 1783-6.

KULL, F.C., BONORDEN, R., and MAYER, R.L. (1954).- Inhibition of melanin formation 'in vivo' by 4-chlororesorcinol. Proc. Soc. Exptl. Biol. Med. 87: 538-40.

KUMMERT, J., and SEMAL, J. (1967).- Mesure de la synthese de virus de plantes par incorporation de precurseurs radioactifs de l'acide ribonucleique. Bull. Rech. Agron. Gembloux, N.S. II: 507-16.

KUNKEL, L.O. (1936).- Heat treatments for the cure of yellows and other virus diseases of peach. Phytopathology 26: 809-30.

KUNKEL, L.O. (1937).- Effect of heat on the ability of Cicadula sexnotata (Fal.) to transmit aster yellows. Am. J. Botany 24: 316-27.

KUNKEL, L.O. (1941).- Heat cure of aster yellows in periwinkles. Am. J. Botany 28: 761-9.

KUNKEL, L.O. (1943).- Potato witches broom transmission and cure by heat. Proc. Am. Phil. Soc. 86: 470-5. (abs. in Rev. appl. Mycol. 23-39).

KUNKEL, L.O. (1945).- Studies on cranberry false blossom. Phytopathology 35: 805-21.

KUNKEL, L.O. (1952).- Transmission of alfalfa witches broom to non-leguminous plants by dodder, and cure in periwinkle by heat. Phytopathology 42: 27-31.

LANGRIDGE, J. (1963).- Biochemical aspects of temperature response. Ann. Rev. Plant Physiol. 14: 441-62.

LAUFFER, M.A., and PRICE, W.C. (1940).- Thermal denaturation of tobacco mosaic virus. J. biol. Chem. 133: 1-15.

LAWSON, R.H. (1967).- Relationships among tomato aspermy, aspermy-related viruses from chrysanthemum, and two strains of cucumber mosaic virus. Virology 32: 357-62.

LEE, S.M., and LIU, H.P. (1961).- Studies on the effect of ratoon stunting disease on the yield of N: Co 310. Rept. Taiwan Sugar Exp. Sta. (Taiwan) 25: 111-8. (abs. in Rev. appl. Mycol. 41: 673).

LIN, K.H. (1964).- A preliminary study on the resistance of yellow shoot virus and citrus budwood tissue to heat. Acta phytopath. sin. 7: 61-5. (abs. in Rev. appl. Mycol. 44: 1092).

LIN, K.H., and LO, H.H. (1965).- A preliminary study on thermotherapy of yellow shoot disease of citrus. Acta phytolac. sin. 4: 169-75. (abs. in Rev. appl. Mycol. 45: 1067).

LINACRE, E.T. (1967).- Further notes on a feature of leaf and air temperatures. Arch. Met. Geophys. Bioklim. 15: 422-36.

LING, K.C., and CHUANG-YANG, C. (1965).- Studies on the white leaf disease of sugar cane. II Efficacy of hot-water treatment in the disease. Taiwan Sugar Exp. Sta. Rep. 30: 69-102 (abs. in Rev. appl. Mycol. 44: 825).

LIU, H.P., LEE, S.M., and TENG, W.S. (1963).- Studies on the ratoon stunting disease of sugarcane. I Effect of one heat treatment on the control of RSD in seed-cane with various years of infection. Rep. Taiwan Sugar Exp. Sta. 32: 131-41. (abs. in Rev. appl. Mycol. 44: 824).

LORING, H.S. (1942).- The reversible inactivation of tobacco mosaic virus by crystalline ribonuclease. J. gen. Physiol. 25: 497-505.

LUGG, J.W.H., and WELLER, R.A. (1944).- On the sulphur present in the seeds of certain legumes, with special reference to the cystine plus cysteine and methionine contents of the proteins. Aust. J. exp. Biol. med. Sci. 22: 149-55.

LWOFF, A. (1969).- Death and transfiguration of a problem. Bacteriol. Rev. 33: 390-403.

LYTTLETON, J.W., and MATTHEWS, R.E.F. (1958).- Release of nucleic acid from turnip yellow mosaic virus under mild conditions. Virology 6: 460-71.

MAJORANA, G. (1966).- Infezioni naturali di 'variegatura infettiva' su Mandarino (Citrus reticulata Blanco). Nota preventiva. Phytopath. Mediterranea 5: 185-7. (abs. in Rev. appl. Mycol. 47: 189).

MAJORANA, G., and MARTELLI, G.P. (1966).- Tentativi di termoterapia di Mandorli affetti da 'mosaico'. Phytopath. Mediterranea 5: 103-8.

MANDELL, J.D., and HERSHEY, A.D. (1960).- A fractionating column for analysis of nucleic acids. Anal. Biochem. 1: 66-77.

MARKHAM, R. (1942).- A steam distillation apparatus suitable for micro-Kjeldahl analysis. Biochem. J. 36: 790-1.

MARKHAM, R. (1960).- A graphical method for the rapid determination of sedimentation coefficients. Biochem. J. 77: 516-9.

MARTIN, J.P. (1933).- Plant pathology. Hawaiian Sugar Planters' Assoc. Exptl. Sta. Ann. Rep., 1932: 23-42. (abs. in Rev. appl. Mycol. 12: 722-3).

MARTYN, E.B. (1968).- "Plant virus names". (Commonwealth Mycological Inst., Phytopathol. Papers, 9, Kew, Surrey, England, 204 pp).

MATTHEWS, R.E.F. (1953).- Factors affecting the production of local lesions by plant viruses. I The effect of time of day of inoculation. Ann. appl. Biol. 40: 377-83.

MATTHEWS, R.E.F., and LYTTLETON, J.W. (1959).- Heat inactivation of turnip yellow mosaic virus in vivo. Virology 9: 332-42.

McLEAN, A.P.D., SCHWARZ, R.E., and OBERHOLZER, P.C.J. (1968).- Greening disease of citrus in South Africa. Proc. Internat. Citrus Symposium, Riverside, Calif., March 1968. (cited by Olson & Rogers, 1969).

MEER, van der, F.A. (1967).- The effect of hot water treatment on a virus of Optunia exaltata. Neth. J. Plant Path. 73: 58-9.

MEGAHED, E., and MOORE, J.D. (1969).- Inactivation of necrotic ringspot and prune dwarf viruses in seeds of some Prunus spp.. Phytopathology 59: 1758-60.

MELLOR, F.C., and FITZPATRICK, R.E. (1961).- Strawberry viruses. Can. Plant Disease Sur. 41: 218-55.

MELLOR, F.C., and STACE-SMITH, R. (1970).- Virus strain differences in eradication of potato viruses X and S. Phytopathology 60: 1587-90.

MILLER, P.W. (1954).- Inactivation of nonpersistent viruses in strawberry plants by hot-air treatments. Plant Disease Reptr. 38: 827-31.

MINK, G.I. (1965).- Inactivation of Tulare apple mosaic virus by o-quinones. Virology 26: 700-7.

MINK, G.I. (1967).- An indirect role of cytochrome oxidase in loss of prune dwarf virus infectivity in squash tissue homogenates. Phytopathology 57: 797-8.

MINK, G.I. (1969).- Serological relationships among cucumber mosaic virus, tomato aspermy type viruses, and peanut stunt virus. Phytopathology 59: 1889-93.

MOLLER, W., AMONS, R., GROENE, J.C.L., GARRETT, R.A., and TERHORST, C.P. (1969).- Protein-ribonucleic acid interactions in ribosomes. Biochim. Biophys. Acta 190: 381-90.

MOORE, S. (1963).- On the determination of cystine in cysteic acid. J. biol. Chem. 238: 235-7.

MOREL, G., and MARTIN, C. (1952).- Guerison de dahlias atteints d'une maladie a virus. C. R. Acad. Sci. Paris 235: 1324-5.

MUHLRAD, A., HEGYI, G., and TOTH, G. (1967).- Effect of diethylpyrocarbonate on proteins. I Reaction of diethylpyrocarbonate with amino acids. Acta Biochim. Biophys. Acad. Sci. Hung. 2: 19-29.

MUKHERJEE, A.K., and RAYCHAUDHURI, S.P. (1966).- Therapeutic treatments against leaf curl of some malvaceous plants. Plant Disease Reptr. 50: 88-90.

MULDER, D. (1963).- Spikiness disease of Guatemala grass (Tripsacum laxum Nash): a virus disease? Tea Quart. 34: 16-8. (abs. in Rev. appl. Mycol. 43: 110).

NAGAICH, B.B. (1963).- Role of thermotherapy in asexually propagated crops. Indian Phytopath. 16: 108. (abs. in Rev. appl. Mycol. 43: 1241h).

NAGAICH, B.B., and UPRETI, G.C. (1964).- Heat inactivation of potato leafroll virus. Indian Potato J. 6: 96-102. (abs. in Rev. appl. Mycol. 45: 174).

NAORA, H., and KODAIRA, K. (1968).- Non-competitive binding of rapidly labeled nuclear RNA by ribosomes. Biochim. Biophys. Acta 161: 276-8.

NICHOLS, C.W., and NYLAND, G. (1952).- Hot water treatment of some stone fruit viruses. Phytopathology 42: 517 (abs.).

NIENHAUS, F., and YARWOOD, C.E. (1963).- Translocated wound stimuli affecting plant virus infections. Virology 20: 477-83.

NORRIS, D.O. (1946).- The strain complex and symptom variability of tomato spotted wilt virus. Counc. Sci. Ind. Res. (Australia) Bull. 202.

NYLAND, G. (1959).- Hot-water treatment of Lambert cherry budsticks infected with necrotic rusty mottle virus. Phytopathology 49: 157-8.

NYLAND, G. (1960).- Heat inactivation of stone fruit ringspot virus. Phytopathology 50: 380-2.

NYLAND, G. (1964).- Thermotherapy of virus-infected fruit trees. Proc. 5th Europ. Symposium on Fruit Tree Virus Diseases: 156-60.

NYLAND, G., and GOHEEN, A.C. (1969).- Heat therapy of virus diseases of perennial plants. Ann. Rev. Phytopath. 7: 331-54.

NYLAND, G., and REEVES, E.L. (1962).- Heat inactivation of the little cherry virus in trees of Shirofugen flowering cherry. Mededel. Landbouwhogeschool. Gent 27: 1060-1 (cited by Hollings, 1965a).

OERTEL, C. (1967).- Serologische Untersuchungen zum Verhalten des Tomatenaspermie - Virus in Chrysanthemum indicum L.. Zentbl. Bakt. ParasitKde 121: 276-86.

OLAND, K. (1959).- Nitrogenous reserves of apple trees. Physiol. Plant. 12: 594-648.

OLIVER, I. (1968).- Factorial analysis of variance. Commun. Assoc. comput. Mach. 11: 431-2.

OLSON, E.O., and ROGERS, B. (1969).- Effects of temperature on expression and transmission of stubborn disease of citrus. Plant Disease Reptr. 53: 45-9.

OS van, H. (1964).- Production of virus-free carnations by means of meristem culture. Neth. J. Plant Pathol. 70: 18-26.

OSBORNE, D.J. (1962).- Effect of kinetin on protein and nucleic acid metabolism in Xanthium leaves during senescence. Plant Physiol. 37: 595-602.

OUCHTERLONY, O. (1962).- Diffusion-in-gel methods for immunological analysis. Progress in allergy 6: 130-54.

PALUDAN, N. (1964).- Inaktiveringsforsøg med virus-inficeret Nellikemateriale. Maanedsovers. PlSygd. 412: 83-8. (abs. in Rev. appl. Mycol. 44: 1110).

PALUDAN, N. (1965).- Inaktiveringsforsøg med Nellike-spaetning-virus. Maanedsovers. PlSygd. 418: 65-8. (abs. in Rev. appl. Mycol. 45: 465).

PARTHIER, B., and WOLLGIEHN, R. (1961).- Uber den Einfluss des Kinetins auf den Eiweissund Nukleinsaure stoffwechsel isolierten Tabakblattern. Ber. Deut. Botan. Ges. 74: 47-51.

PEACOCK, H.A. (1966).- "Elementary microtechnique". (3rd ed.) (Edward Arnold Publishers Ltd.: London) pp. 241-2.

PHILLIPS, I.D.J. (1964a).- Root-shoot hormone relations. I The importance of an aerated root system in the regulation of growth hormone levels in the shoot of Helianthus annuus. Ann. Bot. 28: 17-25.

PHILLIPS, I.D.J. (1964b).- Root-shoot hormone relations. II Changes in endogenous auxin concentration produced by flooding of the root system in Helianthus annuus. Ann. Bot. 28: 37-45.

POLLARD, E.C., and DIMOND, A.E. (1952).- Constitution of two plant viruses as suggested by effects of deuteron bombardment on their infectivity and serology. Phytopathology 42: 472 (abs.).

POSNETTE, A.F. (1953).- Heat inactivation of strawberry viruses. Nature 171: 312.

POSNETTE, A.F. (1961).- Production of virus-free stocks. Proc. Aust. Plant Path. Conf. 7: 1-6.

POSNETTE, A.F., and CROPLEY, R. (1956).- Apple mosaic viruses. Host reactions and strain interference. J. hort. Sci. 31: 119-33.

POSNETTE, A.F., and CROPLEY, R. (1958).- Heat treatment for the inactivation of strawberry viruses. J. hort. Sci. 33: 282-8.

POSNETTE, A.F., CROPLEY, R., and ELLENBERGER, C.E. (1953).- Progress in the heat treatment for strawberry virus diseases. E. Malling Res. Sta. Rept. 1952: 128-30.

POSNETTE, A.F., CROPLEY, R., and WOLFSWINKEL, L.D. (1962).- Heat inactivation of some apple and pear viruses. E. Malling Res. Sta. Rept. 1960-61: 94-6.

POSNETTE, A.F., and ELLENBERGER, C.E. (1963).- Further studies on green petal and other leafhopper transmitted viruses infecting strawberry and clover. Ann. appl. Biol. 51: 69-83.

POSNETTE, A.F., and JHA, A. (1960).- The use of cuttings and heat treatment to obtain virus-free strawberry plants. E. Malling Res. Sta. Rept. 1959: 98.

POUND, G.S. (1949).- The effect of air temperature on virus concentration and leaf morphology of mosaic-infected horseradish. J. agric. Res. 78: 161-70.

PRICE, W.C. (1933).- The thermal death rate of tobacco mosaic virus. Phytopathology 23: 749-69.

PRICE, W.C. (1940).- Thermal inactivation rates of four plant viruses. Arch. fur ges. Virusforsch. 1: 373-86.

PRICE, W.C. (1964).- Strains, mutation, acquired immunity, and interference. in "Plant Virology". (Ed. M.K. Corbett & H.D. Sisler) (University of Florida Press: Gainesville) pp. 93-117.

QUAK, F. (1961).- Heat treatment and substances inhibiting virus multiplication, in meristem culture in order to obtain virus-free plants. Advan. Hort. Sci. appl. 1: 144-8.

RACUSEN, D., and FOOTE, M. (1960).- Amino acid turnover and protein synthesis in leaves. Arch. Biochem. Biophys. 90: 90-5.

RADER, W.E., FITZPATRICK, H.F., and HILDEBRAND, E.M. (1947).- A seed-borne virus of muskmelon. Phytopathology 37: 809-16.

RANDLES, J.W. (1968).- Ribonuclease isozymes in Chinese cabbage systemically infected with turnip yellow mosaic virus. Virology 36: 556-63.

RAYCHAUDHURI, S.P. (1953).- Studies on bayberry yellows. Phytopathology 43: 15-20.

REES, M.W., SHORT, M.N., and KASSANIS, B. (1970).- The amino acid composition, antigenicity, and other characteristics of the satellite viruses of tobacco necrosis virus. Virology 40: 448-61.

REGENMORTEL, van, M.H.V. (1966).- Plant virus serology. Advan. Virus Res. 12: 207-71.

REICH, E., and GOLDBERG, I.H. (1964).- Actinomycin and nucleic acid function. Progr. Nucleic Acid Res. 3: 183-234.

RICH, A.E. (1969).- Inactivation of potato virus X in Green Mountain potatoes. Phytopathology 59: 710-1.

ROSS, A.F. (1966).- Systemic effects of local lesion formation. in "Viruses of plants". (Ed. A.B.R. Beemster & J. Dijkstra) (North-Holland Publishing Co.: Amsterdam) pp. 127-50.

SAKSENA, K.N., and MINK, G.I. (1970).- The effects of oxidised phenolic compounds on the infectivity of four "stable" viruses. Virology 40: 540-6.

SAMPSON, P.J., and TAYLOR, R.H. (1968).- A comparison of the electron microscope, microprecipitin tests, and indicator plants for the detection of potato viruses S, X and Y. Phytopathology 58: 489-93.

SANDER, E. (1969).- Stimulation of biosynthesis of tobacco mosaic virus by antimetabolites. J. gen. Virol. 4: 235-44.

SANGER, H.L., and KNIGHT, C.A. (1963).- Effect of actinomycin D on RNA synthesis in healthy and virus-infected tobacco leaves. Biochem. Biophys. Res. Comm. 13: 455-61.

SCHACHMAN, H.K. (1959).- "Ultracentrifugation in biochemistry". (Academic Press, New York).

SCHIEBEL, W., CHAYKA, T.G., De VRIES, A., and RUSCH, H.P. (1969).- Decrease of protein synthesis and breakdown of polyribosomes by elevated temperature in Physarum polycephalum. Biochem. Biophys. Res. Commun. 35: 338-45.

SCHLEGEL, D.E. (1960).- Transmission of several plant viruses by phenol-water extracts of diseased tissues. Phytopathology 50: 156-8.

SCHNEIDER, I.R. (1953).- Solution of tobacco mosaic virus in the aqueous phase of a chloroform-water emulsion and application of this phenomenon in virus assay. Science N.Y. 117: 30-1.

SCHNEIDER, I.R., and DIENER, T.O. (1968).- 'In vivo' and 'in vitro' decline of specific infectivity of tobacco ringspot virus correlated with nucleic acid degradation. Virology 35: 150-7.

SCHROEDER, C.A. (1963).- Induced temperature tolerance of plant tissue in vitro. Nature 200: 1301-2.

SCOTT, H.A. (1963).- Purification of cucumber mosaic virus. Virology 20: 103-6.

SELSKY, M.I. (1960).- Factors affecting the occurrence of symptomless and virus-free plants propagated from wound-tumour sweet clover seedlings. Dissertation Abstr. 20: 4485.

SEMAL, J. (1967).- Effects of actinomycin D in plant virology. Phytopath. Z. 59: 55-71.

SEMANCIK, J.S., and WEATHERS, L.G. (1968).- Exocortis virus of citrus; association of infectivity with nucleic acid preparations. Virology 36: 326-8.

SHINDE, B.G., CHANDRASEKHAR, B.K., and SANTILLI, V. (1964).- Distribution of ribonuclease in subcellular fractions of untreated, wounded, and TMV infected Pinto bean leaves. Phytopathology 54: 908.

SHINDE, B.G., and SANTILLI, V. (1967).- Effect of actinomycin D on the tobacco mosaic virus infection-induced increase in ribonuclease activity. Phytopathology 57: 345.

SHIROYA, M., SHIROYA, T., and HATTORI, S. (1955).- Studies on the browning and blackening of plant tissues. IV Chlorogenic acid in the leaves of Nicotiana tabacum. Physiol. Plant. 8: 594-605.

SIEGEL, A. (1966).- The first stages of infection. in "Viruses of plants". (Ed. A.B.R. Beemster & J. Dijkstra) (North-Holland Publishing Co.: Amsterdam) pp. 3-18.

SINGH, K. (1967).- Heat therapy of sugarcane. Indian Sug. 17: 181-6. (abs. in Rev. appl. Mycol. 46: 3555).

SINHA, R.C. (1967).- Response of wound tumour infection in insects to vector age and temperature. Virology 31: 746-8.

SIP, V. (1965).- Pokusy s termoterapii u viru svinutky Brambor. Ochr. Rost. 1: 35-42. (abs. in Rev. appl. Mycol. 45: 1131).

SISLER, E.C., and EVANS, H.J. (1958).- A comparison of chlorogenic acid and catechol as substrates for the polyphenol oxidase from tobacco and mushroom. Plant Physiol. 33: 255-7.

SMITH, K.M. (1957).- "A textbook of plant virus diseases". (2nd ed.) (J. and A. Churchill, London).

SNEDECOR, G.W. (1956).- "Statistical methods applied to experiments in agriculture and biology". (Iowa State College Press) (5th edn.).

SOANS, L.C. (1967).- Effect of some cytokinins on tobacco and their relation to tobacco mosaic virus infection. Diss. Abstr. 27B: 4210-1.

SOLYMOSY, F., FEDORCSAK, I., GULYAS, A., FARKAS, G.L., and EHRENBERG, L. (1968).- A new method based on the use of diethyl pyrocarbonate as a nuclease inhibitor for the extraction of undegraded nucleic acid from plant tissues. European J. Biochem. 5: 520-7.

SRIVASTAVA, B.I.S., and WARE, G. (1965).- The effect of kinetin on nucleic acids and nucleases of excised barley leaves. Plant Physiol. 40: 62-4.

STACE-SMITH, R., and MELLOR, F.C. (1968).- Eradication of potato viruses X and S by thermotherapy and axillary bud culture. Phytopathology 58: 199-203.

STEERE, R.L. (1952).- Virus increment curves obtained from counts of particles in clarified plant juice. Amer. J. Bot. 39: 211-220.

STEERE, R.L. (1959).- The purification of plant viruses. Advan. Virus Res. 6: 1-73.

STEIB, R.J., and FORBES, I.L. (1958).- Hot air for the control of the ratoon stunting disease of sugarcane in Louisiana. Phytopathology 48: 398 (abs.).

STEIB, R.J., THAUNG, M.M., and WANG, L. (1954).- Effect of heat treatment tests on the germination and yields of sugarcane. Phytopathology 44: 507. (abs.).

STEINDL, D.R.L., and HUGHES, C.G. (1953).- Ratoon stunting disease. Cane Growers' Quart. Bull. 16: 79-95.

STENESH, J., and HOLAZO, A.A. (1967).- Studies of the ribosomal ribonucleic acid from mesophilic and thermophilic bacteria. Biochim. Biophys. Acta 138: 286-95.

STENT, G.S. (1964).- The operon: on its third anniversary. Science N.Y. 144: 816-20.

STODDARD, E.M. (1942).- Inactivating 'in vivo' the virus of X-disease of peach by chemotherapy. Phytopathology 32: 17 (abs.).

STUBBS, L.L. (1963a).- Production of virus free propagating material. Vic. J. Agric. 61: 421-8.

STUBBS, L.L. (1963b).- A phytotron cabinet for heat-therapy studies with virus-infected plants. Comm. Phytopath. News 9: 49-52.

STUBBS, L.L. (1966).- Inactivation of viruses in plants grown in a high-temperature environment. Proc. Aust. Plant Path. Conf. 1: 11-16.

STUBBS, L.L. (1968).- Apparent elimination of exocortis and yellowing viruses in lemon by heat therapy and shoot-tip propagation. "Proc. 4th Conf. Intern. Organ. Citrus Virologists, Italy": 96-9.

TAHAMA, Y. (1964).- Studies on the dwarf disease of mulberry tree. VIII Recovery by heat treatment. Ann. Phytopathol. Soc. Japan 29: 39-42 (abs. in Rev. appl. Mycol. 43: 2080).

TAYLOR, R.H. (1959).- An investigation of the viruses which cause woodiness of passionfruit. J. Australian Inst. Agric. Sci. 25: 71.

THIRUMULACHAR, M.J. (1954).- Inactivation of potato leafroll by high temperature storage of seed tubers in Indian plains. Phytopathol. Z. 22: 429-36.

THOMAS, R.G. (1961).- Correlations between growth and flowering in Chenopodium amaranticolor. II Leaf and stem growth. Ann. Bot. N.S. 25: 255-69.

THOMSEN, A. (1968).- Frugttrae-vira inaktiveret ved termoterapi. Tidsskr. Planteavl. 72: 141-52. (abs. in Rev. appl. Mycol. 48: 513).

THOMSON, A.D. (1956).- Studies on the effect of malachite green on potato viruses X and Y. Aust. J. agric. Res. 7: 428-34.

THOMSON, A.D. (1958).- The elimination of viruses from potato tissue. Proc. 3rd Conf. Potato Virus Diseases, Lisse-Wageningen, 1957: 156-9.

THORNBERRY, H.H. (1935).- Effect of phosphate buffers on infectivity of tobacco mosaic virus. Phytopathology 25: 618-27.

THUNG, T.H. (1952).- Warnemingen omtrent de dwergziekte bij framboos en wild braam. Tidschr. Plantenziekten 58: 255-9. (abs. in Rev. appl. Mycol. 32: 683).

TRAYLOR, J.A., WILLIAMS, H.E., and NYLAND, G. (1967).- Heat therapy of rose mosaic. Phytopathology 57: 1010 (abs.).

TSOU, P., JUO, P., and RICH, A.E. (1967).- Inhibition of potato virus X by a naturally occurring substance in immune variety Saco. Phytopathology 57: 345. (abs.).

VALENTA, V. (1959).- Pokusy s tepelnou inaktivaciou niektorych europskych zltackovych virusov in vivo. Biologia (Bratislava) 14: 146-8.

VALENTA, V. (1962).- Thermal inactivation of yellows-type viruses 'in vivo'. Acta Virol. 6: 94. (abs. in Rev. appl. Mycol. 43: 56).

VINE, S.J., and JONES, O.P. (1969).- The culture of shoot tips of hop (Humulus lupulus L.) to eliminate viruses. J. hort. Sci. 44: 281-4.

VOLD, B.S., and SYPHERD, P.S. (1968).- Modification in transfer RNA during differentiation of wheat seedlings. Proc. Natl. Acad. Sci. U.S. 59: 453-8.

WADE, G.C., WARD, J.R., GEARD, I.D., CARTLEDGE, E.G., WILLIAMS, P.G., and SAMPSON, P.J. (1959).- A list of plant diseases recorded in Tasmania. Tasm. J. agric. Res. Bull. 2.

WALKEY, D.G.A., and WEBB, M.J.W. (1968).- Virus in apical meristems. J. gen. Virol. 3: 311-3.

WEISSMANN, C., BILLETER, M.A., VINUELA, E., and LIBONATI, M. (1966).- Double-stranded RNA and the replication of viral RNA. in "Viruses of plants". (Ed. A.B.R. Beemster & J. Dijkstra) (North-Holland Publishing Co.: Amsterdam) pp. 249-74.

WELSH, M.F., and NYLAND, G. (1965).- Elimination and separation of viruses in apple clones by exposure to dry heat. Can. J. Plant Sci. 45: 443-54.

WHITE, N.H. (1968).- The local lesion reaction and resistance in plants to systemic virus disease. Aust. J. Sci. 31: 223-5.

WIEHE, P.O. (1966).- The control of sugar cane diseases in Mauritius. Sugar J. 28: 41-2 (abs. in Rev. appl. Mycol. 45: 3210).

WILBRINK, G. (1923).- Warmwaterbehandeling van stekken als geneesmiddel tegen de serehziekte van het suikerriet. Arch. Suikerind. Ned.-Ind. 31: 1-15.

WOODHAM, R.C. (1967).- Viruses of grape vines. C.S.I.R.O. Division of Horticultural Res. Rept. 1965-7: 14-16.

WU, J.H., and RAPPAPORT, I (1961).- Kinetic study of heat inactivation of tobacco mosaic virus infected centres and potentially infectible sites on Nicotiana glutinosa. Phytopathology 51: 823-6.

WYEN, N.V., UDVARDY, J., SOLYMOSY, F., MARRE, E., and FARKAS, G.L. (1969).- Purification and properties of a ribonuclease from Avena leaf tissues. Biochim. Biophys. Acta 191: 588-97.

YARWOOD, C.E. (1957).- Mechanical transmission of plant viruses. Advan. Virus Res. 4: 243-78.

YARWOOD, C.E. (1961).- Acquired tolerance of leaves to heat. Science 134: 941-2.

YARWOOD, C.E., and HOLM, E.W. (1962).- Heat adaptation in a rust and a virus. Phytopathology 52: 709-12.

YARWOOD, C.E., RESCONICH, E.C., and KADO, C.I. (1962).- Translocated stimuli affecting plant virus infections. Virology 16: 414-8.

YOUDEN, W.J., and BEALE, H.P. (1934).- A statistical study of the local lesion method for estimating tobacco mosaic virus. Contrib. Boyce Thompson Inst. 6: 437-54.

ZEIKUS, J.G., TAYLOR, M.W., and BROCK, T.D. (1970).- Thermal stability of ribosomes and RNA from Thermus aquaticus. Biochim. Biophys. Acta 204: 512-20.

ZUCKER, M., and AHRENS, J.F. (1958).- Quantitative assay of chlorogenic acid and its pattern of distribution within tobacco leaves. Plant Physiol. 33: 246-9.

APPENDICES.

APPENDIX 1: A SUMMARY OF WORK ON HEAT TREATMENT IN AUSTRALIA

In November 1968, organisations thought likely to be using heat treatment in Australia were contacted. Summaries of their replies outlining equipment available, past successes, and future intentions are set out below. They indicate the considerable amount of money, time and effort which has been spent on heat treatment of virus infected plants in Australia.

I am grateful to the heads of the organisations contacted for so willingly supplying the information requested.

(a) Commonwealth Government Departments

(i) C.S.I.R.O. Division of Horticultural Research: a small number of virus-infected sultana varieties were treated during the past three years. Success was limited due to poor equipment, but plans are in hand to purchase a standard growth cabinet so that the work may be expanded.

(ii) Department of Health: a phytotron cabinet was recently purchased for heat therapy work, and several apples, grapes and grasses have been treated. The aim is to evaluate the possibility of using heat treatment in conjunction with the Department's quarantine activities.

(b) State Government Departments

(i) Queensland Department of Primary Industries: a small naturally-lit cabinet has been in use for five years to treat infected clones of apple, pear, strawberry and sweet potato. Strawberry varieties were freed of latent A, mottle and a NEPO virus, and apple varieties of chlorotic leaf spot, mosaic, rubbery wood, Spy decline

and stem pitting. An improved cabinet is to be purchased shortly and heat treatment extended to citrus.

(ii) New South Wales Department of Agriculture: a small room was adapted about four years ago to treat virus-infected clones of apple, citrus, grapevine and strawberry. The only success to date has been the production of virus free Kendall strawberry plants. Future work will aim to obtain healthy strawberry and grapevine clones.

(iii) South Australia Department of Agriculture: it is intended to commence heat treating grapevines in the near future.

(iv) Tasmanian Department of Agriculture: two phytotron cabinets plus a home-made cabinet have been used for several years to treat infected apples, hops and potatoes. Several apple varieties were freed of mosaic and rubbery wood, but less success was achieved with eliminating the latent viruses. Necrotic ringspot was eliminated from hop and the main commercial potato varieties were freed of viruses S, X and Y when heat treatment was combined with meristem culture. Future work will aim to eliminate latent viruses from apple.

(v) Victorian Plant Research Institute: two phytotron cabinets have been in use for about ten years to treat infected clones of apple, cherry, citrus, grapevine, peach, pear, strawberry and sweet potato. Several healthy varieties of each of the deciduous fruits are now available. The viruses successfully eliminated were

apple mosaic, apple rubbery wood, apple flat limb, apple chlorotic leafspot, apple stem grooving, apple stem pitting, Spy decline, pear mosaic, quince sooty ringspot, peach greasy mottle, Prunus necrotic ringspot and prune dwarf. Clones of several citrus fruits were freed of tristeza and exocortis, clones of grapevine freed of fanleaf, and clones of strawberry freed of crinkle, mottle, and vein banding. Some imported sweet potatoes were freed of the feathery mottle complex. Further heat treatment of fruit crops will continue as the need arises, but the present aim is to obtain healthy material of the vegetatively propagated flower crops.

(vi) Western Australia Department of Agriculture: several apple varieties were freed of mosaic between 1960 and 1964, when further work on heat treatment was discontinued indefinitely.

(c) Universities

(i) University of Adelaide: some work was undertaken on heat treatment of deciduous fruits a few years ago. A cabinet is available for heat treatment, and the programme may recommence in the near future.

(d) Other Institutions

(i) Bureau of Sugar Experiment Stations: the Cane Pest and Disease Control Boards, ancillary organisations of the Bureau, operate 22 hot water tanks to treat up to 3,000 tons of sugar cane sticks and setts annually. The treatment is carried out at 50°C for three hours and gives

99% control in diseased material. Routine selection of near disease-free material greatly reduces the percentage of infection surviving the treatment.

APPENDIX 2

Mean monthly minimum and maximum temperatures for 1967-70 in the open ventilated glasshouse equipped with strip wall heaters.

Month	Minimum (^{o}F)	Maximum (^{o}F)
January	60.2	82.1
February	59.0	80.7
March	59.6	79.4
April	60.4	73.8
May	56.0	71.5
June	53.3	66.9
July	51.8	67.7
August	56.0	70.8
September	57.3	75.3
October	60.1	79.5
November	58.8	78.8
December	60.5	80.8

APPENDIX 3 (a): retransformed mean fresh weights (g).

Infection	Environment	Spray	Days After Commencing Heat Treatment 5	10	15	20	Mean
healthy	glasshouse	kinetin	4.3	6.8	9.2	8.5	6.9
		benzyladenine	3.5	6.3	8.7	12.6	6.6
		control	3.9	5.6	7.8	10.0	6.4
		mean	3.9	6.1	8.4	9.6	6.6
healthy	cabinet	kinetin	3.5	5.8	6.0	9.6	5.9
		benzyladenine	4.3	4.3	5.9	6.3	5.1
		control	2.7	5.2	5.7	6.5	4.8
		mean	3.4	5.1	5.8	7.3	5.2
infected	glasshouse	kinetin	3.7	4.9	6.4	6.1	5.2
		benzyladenine	4.0	5.6	6.9	6.6	5.6
		control	4.0	4.8	6.0	5.1	4.9
		mean	3.9	5.1	6.4	5.9	5.2
infected	cabinet	kinetin	5.1	5.9	7.5	8.5	6.6
		benzyladenine	4.5	4.2	5.7	6.0	5.0
		control	4.3	5.0	7.9	6.4	5.7
		mean	4.6	5.0	7.0	6.9	5.8

APPENDIX 3 (a) continued:

analysis of variance of transformed fresh weights

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	4	307,766	53.9	< 0.001
infections (I)	1	54,120	9.5	< 0.01
environments (E)	1	60,738	10.6	< 0.01
sprays (S)	2	59,108	10.4	< 0.001
IxE	1	162,141	28.4	< 0.001
IxS	2	2,669	0.5	-
ExS	2	50,306	8.8	< 0.01
IxExS	2	25,680	4.5	< 0.05
error	44	5,707		
times (T)	3	894,603	360.4	< 0.001
error	12	2,482		
IxT	3	84,958	20.3	< 0.001
error	12	4,191		
ExT	3	16,907	17.4	< 0.001
error	12	970		
SxT	6	5,373	1.6	-
error	24	3,412		
IxExT	3	12,489	6.6	< 0.01
error	12	1,897		
IxSxT	6	5,491	2.3	-
error	24	2,370		
ExSxT	6	29,639	7.6	< 0.001
error	24	3,913		
IxExSxT	6	9,139	3.9	< 0.01
error	24	2,361		

APPENDIX 3 (b): retransformed mean dry weights (g).

Infection	Environment	Spray	Days After Commencing Heat Treatment 5	10	15	20	Mean
healthy	glasshouse	kinetin	0.43	0.80	1.11	1.24	0.87
		benzyladenine	0.38	0.73	1.10	1.48	0.88
		control	0.40	0.59	0.96	1.44	0.81
		mean	0.41	0.70	1.05	1.38	0.85
healthy	cabinet	kinetin	0.26	0.40	0.53	0.93	0.51
		benzyladenine	0.32	0.31	0.45	0.55	0.40
		control	0.23	0.33	0.50	0.46	0.37
		mean	0.27	0.34	0.49	0.63	0.43
infected	glasshouse	kinetin	0.43	0.71	0.96	1.06	0.77
		benzyladenine	0.45	0.76	0.86	1.11	0.78
		control	0.46	0.65	0.81	0.90	0.70
		mean	0.44	0.70	0.88	1.02	0.75
infected	cabinet	kinetin	0.41	0.39	0.55	0.78	0.52
		benzyladenine	0.35	0.39	0.43	0.55	0.43
		control	0.36	0.38	0.51	0.45	0.42
		mean	0.38	0.38	0.50	0.59	0.46

APPENDIX 3 (b) continued:

analysis of variance of transformed dry weights

Source of Variance	d.f.	Variance	F Ratio	Significance
replicates	4	39,477	40.2	<0.001
infections (I)	1	3,650	3.7	-
environments (E)	1	550,467	561.7	<0.001
sprays (S)	2	13,721	14.0	<0.001
IxE	1	17,069	17.4	<0.001
IxS	2	60	0.1	-
ExS	2	4,898	5.0	<0.05
IxExS	2	279	0.3	-
error	44	980		
times (T)	3	212,511	191.5	<0.001
error	12	1,108		
IxT	3	12,322	23.6	<0.001
error	12	523		
ExT	3	30,142	76.3	<0.001
error	12	395		
SxT	6	1,970	2.7	<0.05
error	24	730		
IxExT	3	1,563	1.9	-
error	12	823		
IxSxT	6	966	1.6	-
error	24	597		
ExSxT	6	5,519	5.3	<0.01
error	24	1,046		
IxExSxT	6	1,438	1.8	-
error	24	798		

APPENDIX 3 (c): moisture content (% F.W.)

Infection	Environment	Spray	Days After Commencing Heat Treatment 5	10	15	20	Mean
healthy	glasshouse	kinetin	90.1	88.2	87.9	85.6	87.9
		benzyladenine	89.2	88.5	87.5	85.2	87.6
		control	90.1	89.5	87.7	85.7	88.2
		mean	89.8	88.7	87.7	85.5	87.9
healthy	cabinet	kinetin	92.8	93.6	91.4	90.6	92.1
		benzyladenine	92.7	93.5	92.4	91.7	92.6
		control	92.4	93.8	91.6	92.9	92.7
		mean	92.6	93.6	91.8	91.7	92.5
infected	glasshouse	kinetin	88.5	85.6	85.1	82.6	85.4
		benzyladenine	88.8	86.6	86.3	83.6	86.3
		control	88.5	86.6	86.3	82.4	85.9
		mean	88.6	85.9	85.9	82.9	85.9
infected	cabinet	kinetin	91.9	93.5	92.7	91.0	92.3
		benzyladenine	92.2	91.8	92.5	91.0	91.9
		control	91.8	92.4	93.7	93.6	92.9
		mean	92.0	92.2	93.0	91.9	92.4

APPENDIX 3 (c) continued:

analysis of variance of moisture contents

Source of Variance	d.f.	Variance	F Ratio	Significance
replicates	4	2.16	1.4	-
infections (I)	1	67.52	44.6	<0.001
environments (E)	1	1796.90	1186.2	<0.001
sprays (S)	2	5.17	3.4	<0.05
IxE	1	57.14	37.7	<0.001
IxS	2	0.13	0.1	-
ExS	2	1.01	0.7	-
IxExS	2	6.59	4.4	<0.05
error	44	1.51		
times (T)	3	88.70	71.5	<0.001
error	12	1.24		
IxT	3	5.44	2.9	-
error	12	1.85		
ExT	3	50.35	36.1	<0.001
error	12	1.40		
SxT	6	1.66	1.0	-
error	24	1.61		
IxExT	3	4.73	2.4	-
error	12	1.98		
IxSxT	6	1.15	1.4	-
error	24	0.83		
ExSxT	6	4.37	3.7	<0.01
error	24	1.19		
IxExSxT	6	0.42	0.2	-
error	24	1.78		

APPENDIX 3 (d): retransformed mean total nitrogen content (% D.W.)

Infection	Environment	Spray	Days After Commencing Heat Treatment 5	10	15	20	Mean
healthy	glasshouse	kinetin	3.7	2.4	2.4	1.8	2.5
		benzyladenine	3.2	2.3	2.5	1.8	2.4
		control	3.8	2.8	2.5	1.6	2.6
		mean	3.6	2.5	2.5	1.8	2.5
healthy	cabinet	kinetin	5.1	4.5	3.5	2.7	3.8
		benzyladenine	3.5	4.4	3.1	3.4	3.6
		control	3.5	4.1	2.8	3.4	3.4
		mean	4.0	4.3	3.1	3.2	3.6
infected	glasshouse	kinetin	2.3	1.6	1.4	1.1	1.5
		benzyladenine	2.2	1.7	1.5	1.2	1.6
		control	2.1	1.6	1.4	1.2	1.6
		mean	2.2	1.7	1.4	1.1	1.6
infected	cabinet	kinetin	3.0	3.6	3.2	2.9	3.1
		benzyladenine	3.0	2.8	2.7	2.3	2.7
		control	3.6	3.7	4.1	4.3	3.9
		mean	3.2	3.3	3.3	3.1	3.2

APPENDIX 3 (d) continued:

analysis of variance of transformed total nitrogen contents

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	4	.027	5.5	<0.01
infections (I)	1	.945	190.1	<0.001
environments (E)	1	3.370	678.1	<0.001
sprays (S)	2	.033	6.6	<0.01
IxE	1	.341	68.5	<0.001
IxS	2	.034	6.8	<0.01
ExS	2	.022	4.5	<0.05
IxExS	2	.062	12.6	<0.001
error	44	.005		
times (T)	3	.349	70.1	<0.001
error	12	.005		
IxT	3	.010	2.0	-
error	12	.005		
ExT	3	.148	18.7	<0.001
error	12	.008		
SxT	6	.010	1.9	-
error	24	.005		
IxExT	3	.027	3.9	<0.05
error	12	.007		
IxSxT	6	.012	6.4	<0.001
error	24	.002		
ExSxT	6	.010	2.5	-
error	24	.004		
IxExSxT	6	.009	2.1	-
error	24	.004		

APPENDIX 4 (a): analysis of variance of leaf lengths (mm)

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	4	506	2.30	-
environments (E)	1	1171	5.33	< 0.05
infections (I)	1	6757	30.73	< 0.001
ExI	1	54	0.25	-
error	12	220		
times (T)	4	2921	12.63	< 0.001
error	16	231		
TxE	4	47	0.29	-
error	16	161		
TxI	4	80	0.65	-
error	16	122		
TxExI	4	147	0.77	-
error	16	190		

APPENDIX 4 (b): analysis of variance of leaf widths (mm)

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	4	40	0.48	-
environments (E)	1	111	1.34	-
infections (I)	1	1723	20.82	< 0.001
ExI	1	5	0.06	-
error	12	83		
times (T)	4	1036	18.76	< 0.001
error	16	55		
TxE	4	17	0.46	-
error	16	36		
TxI	4	59	1.59	-
error	16	37		
TxExI	4	29	0.33	-
error	16	85		

APPENDIX 5: mitotic index

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	4	0	0.00	-
environments (E)	1	263	210.40	< 0.001
infections (I)	1	0	0.00	-
ExI	1	2	1.60	-
error	12	1		
times (T)	4	23	37.20	< 0.001
error	16	1		
TxE	4	10	7.81	< 0.001
error	16	1		
TxI	4	1	0.80	-
error	16	1		
TxExI	4	1	0.80	-
error	16	1		

APPENDIX 6: DILUTION HEATING PROOFS

(a) Zero Order Reaction

general formula*: $C = C_o - k_o t$

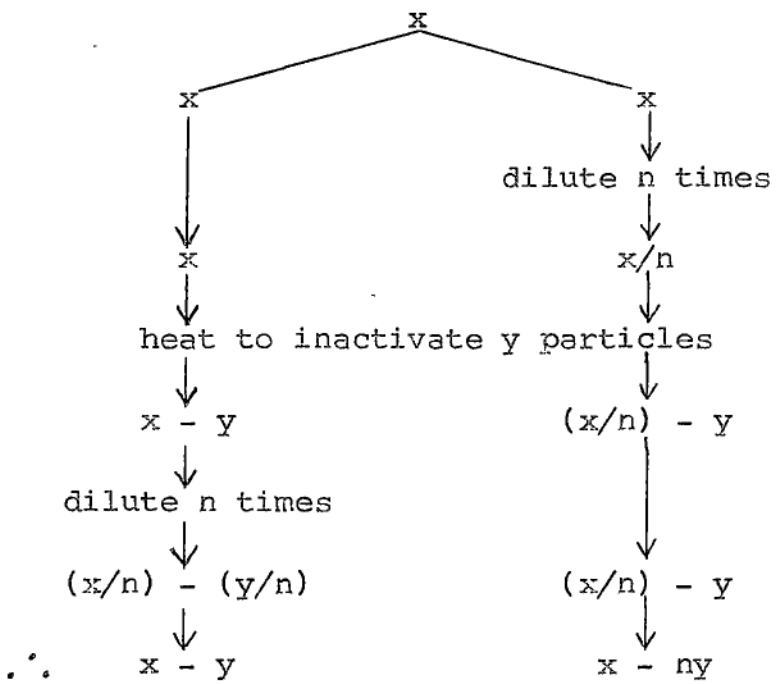
where C = virus concentration at time t

C_o = initial virus concentration

k_o = velocity constant of inactivation

meaning in words*: the number of particles inactivated at any instant is independent of the virus concentration.

dilution heating proof



but $n > 1$

$\therefore$ LHS $>$ RHS

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* "General formulae" and "meaning in words" are taken from Price (1940).

APPENDIX 6 (continued)

(b) First Order Reaction

general formula: $\log_e = \log_e C_o - k_1 t$

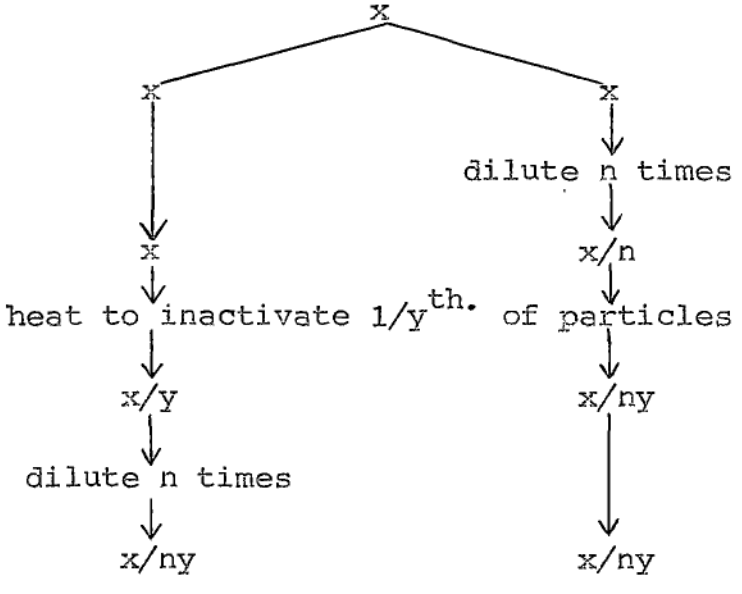
where C = virus concentration at time t

C_o = initial virus concentration

k_1 = velocity constant of inactivation

meaning in words: the number of particles inactivated at any instant is directly proportional to the infective virus concentration.

dilution heating proof



$\therefore$ LHS = RHS

APPENDIX 6 (continued)

(c) Second Order Reaction

general formula: $1/C = -1/C_o + k_2 t$

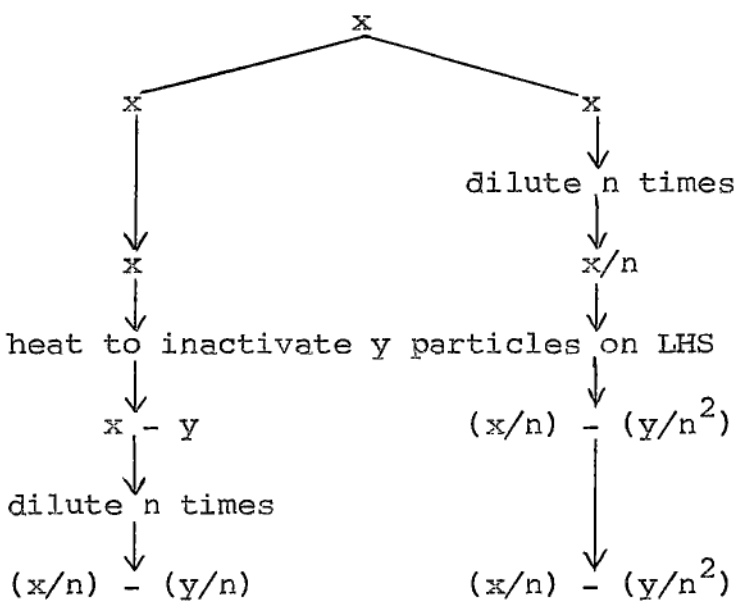
where C = virus concentration at time t

C_o = initial virus concentration

k_2 = velocity constant of inactivation

meaning in words: the number of particles inactivated at any instant is directly proportional to the square of the infective virus concentration.

dilution heating proof



but $n > 1$

$\therefore$ LHS $<$ RHS

APPENDIX 7: polyphenoloxidase content

(a) $[3 + \log(x + 0.001)]$ conversion for days 3-15.

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	2	0.0023	0.06	-
infections (I)	1	0.4344	11.36	< 0.05
error	2	0.0383		
times (T)	4	0.0949	2.49	-
error	8	0.0382		
TxI	4	0.0487	1.06	-
error	8	0.0462		

(b) $[3 + \log(x + 0.001)]$ conversion for days 18-30.

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	2	0.0952	3.69	-
environments (E)	1	14.9101	577.91	< 0.001
infections (I)	1	0.0844	3.27	-
ExI	1	0.0770	2.98	-
error	6	0.0258		
times (T)	4	1.1330	27.97	< 0.001
error	8	0.0405		
TxE	4	0.1304	5.11	< 0.05
error	8	0.0255		
TxI	4	0.0363	1.55	-
error	8	0.0234		
TxExI	4	0.0083	2.20	-
error	8	0.0038		

APPENDIX 8: chlorogenic acid content

(a) for days 3-15.

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	1	0.0006	0.05	-
infections (I)	1	0.0070	0.56	-
error	1	0.0124		
times (T)	4	0.0681	4.13	-
error	4	0.0165		
TxI	4	0.0054	0.39	-
error	4	0.0137		

(b) for days 18-30.

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	1	0.0007	0.21	-
environments (E)	1	1.9391	575.40	< 0.001
infections (I)	1	0.0016	0.48	-
ExI	1	0.0002	0.05	-
error	3	0.0034		
times (T)	4	0.3759	12.51	< 0.05
error	4	0.0301		
TxE	4	0.4398	19.59	< 0.01
error	4	0.0225		
TxI	4	0.0207	1.22	-
error	4	0.0169		
TxExI	4	0.0099	0.16	-
error	4	0.0609		

APPENDIX 9: pH of sap extracts

(a) for infected plants

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	4	0.02825	2.55	-
environments (E)	1	2.18460	196.81	< 0.001
error	4	0.01110		
times (T)	7	0.04660	6.60	< 0.001
error	28	0.00706		
TxE	7	0.04827	9.55	< 0.001
error	28	0.00505		

(b) for healthy plants

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	4	0.02088	1.35	-
environments (E)	1	0.95500	61.91	< 0.001
error	4	0.01543		
times (T)	4	0.04048	9.45	< 0.001
error	16	0.00428		
TxE	4	0.03658	5.79	< 0.01
error	16	0.00632		

APPENDIX 10: Ionic Strength of Sap Extracts

Source of Variation	d.f.	Variance	F Ratio	Significance
replicates	2	0.015959	6.912	< 0.05
environments (E)	1	0.444098	192.333	< 0.001
infections (I)	1	0.002655	1.150	-
ExI	1	0.000285	0.357	-
error	6	0.002309		
times (T)	3	0.027401	3.856	-
error	6	0.007107		
TxE	3	0.042419	13.360	< 0.01
error	6	0.003175		
TxI	3	0.003173	1.862	-
error	6	0.001704		
TxExI	3	0.004722	0.945	-
error	6	0.004996		